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LXXXI. NOTE ON THE FERMENTATION OF *i*-INOSITOL.

By JAMES ARTHUR HEWITT and DOROTHY BEATTY STEABBEN.

From the Department of Physiology, University of London, King's College.

(*Received October 20th, 1921.*)

In the course of other work on the metabolism of carbohydrates it was found desirable to know whether there was any inter-relationship, under normal conditions, between inositol and glucose. Under pathological conditions, such as in phloridzinised dogs, Greenwald and Weiss [1917] have shown that inositol administered is quantitatively excreted as glucose, while in diabetes insipidus it has been found by Strauss [1870], Külz [1875] and McIlraith [1892] in considerable quantity in the urine.

As a preliminary to the investigation referred to, the literature was searched for work on the decomposition of inositol by micro-organisms, as it was thought that this might throw light on some of the possible reactions concerned. Despite the common use in bacteriology of this substance as a test medium, no information could be obtained other than "acid and gas." The general impression appears to be however that lactic acid is the principal product formed.

On carrying out on a small scale some preliminary fermentations of inositol, employing *Bacillus lactis aerogenes*, we were somewhat surprised to be unable to obtain evidence of the presence of this acid, though the reaction mixture was markedly acid and the organism active. Another and larger experiment was planned, but before this was carried out we became aware of some previous unpublished work of Professor Harden in this connection. As our experiment would of necessity have been on a smaller scale than Professor Harden's, it was likely that our analysis would have been less accurate, and it was considered needless to repeat it. Professor Harden has kindly consented to his figures being quoted here, attention being drawn to the fact that, owing to no good method being available, any residual inositol was not determined.

10 g. inositol gave:—

Alcohol	2·33 g.
Acetic acid	1·43 „
Formic „	0·10 „
Lactic „	0·54 „
Succinic „	2·22 „
CO_2	1·92 „
Hydrogen also evolved.	
Total weight of products ...	8·54 „

In view of the apparent lack of definite information on this point it is thought that the figures are worthy of record.

Regarding the formation of glucose as an intermediate product in the fermentation of inositol, it may at once be said that all observations have yielded negative results. Fermentations of inositol by this bacillus, alone, in peptone broth, and in asparagine-salt mixtures have failed to give products among which glucose could be detected. Polarimetric observations also showed, in the reaction mixtures, no greater optical activity than in the controls. By the employment of the "fixation" method of Neuberg [1920], we have failed to obtain any product which could be isolated as a hydrazone by the use of *p*-nitrophenylhydrazine, or phenylhydrazine, and it is concluded that acetaldehyde is not formed. This observation, together with that of Neuberg [1920] that succinic acid is not formed from glucose by *Bacillus lactis aerogenes*, would appear to be evidence against the opening of the reduced hydroxybenzene ring with the formation of glucose as an intermediate product.

The work is incomplete, but, as we are unable to continue it in the immediate future, it is communicated in this note.

The expenses of this research have been defrayed by a grant to one of the authors from the Government Grant Committee of the Royal Society.

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LXXXII. THE METABOLISM OF CARBOHYDRATES. PART II.

ON THE POSSIBLE OCCURRENCE OF STEREOCHEMICAL CHANGES IN EQUILIBRATED SOLUTIONS OF REDUCING SUGARS INTRODUCED INTO THE CIRCULATION.

By JAMES ARTHUR HEWITT and DAVID HENRIQUES DE SOUZA.

From the Department of Physiology, University of London, King's College.

(*Received October 20th, 1921.*)

In view of the existence of stereoisomerides of glucose [Fischer 1914, Irvine, Fyffe and Hogg 1915], fructose [Irvine and Robertson 1916] and galactose [Hudson 1915] much more reactive than the crystalline α- or β-modifications, and the recognition of the formation of certain of these substances under the influence of the living intact intestinal wall either prior to or during absorption [Hewitt and Pryde 1920], it became a matter of interest to determine whether any similar isomeric change occurred in these hexoses when introduced into the circulation by routes other than the alimentary canal[1]. It was thought that by investigating this point information might be obtained on such questions as the condition of glucose in the blood and on the function of γ-sugars in carbohydrate metabolism. It may be presumed on *a priori* grounds that a structural change of the nature referred to has some fundamental reason and the relation of glucose to glycogen has therefore also to be considered.

The scheme of research formulated included:—(1) Observations on the stereochemical condition of certain hexoses as soon as possible after removal from the circulating blood of anaesthetised, but otherwise normal animals; (2) similar observations on the condition of glucose formed *in vivo* by hydrolysis of glycogen. It is the first part of the work which is now communicated.

In view of the rapidity of the change from the γ- form to the equilibrium mixture of α- and β-glucoses, and further owing to the necessity for avoiding as far as possible chemical manipulations, the only method suitable for the purpose seemed to be the removal of the sugar from the blood by the animal's own kidneys and its collection in the urine secreted. By simple filtration, material could be obtained sufficiently clear for the determination of optical activity in a 2 dcm. tube if amount permitted, or otherwise in a 1 dcm. tube. The general technique consisted in intravenous injection into rabbits or dogs

[1] It may be recalled that the last authors could find no evidence for assuming such a change to take place in glucose introduced into the peritoneal cavity

of glucose, fructose or galactose, collection of the urine secreted in the following five minutes and examination of its optical behaviour. In each case the constant optical value was subsequently determined after addition of a trace of caustic alkali. As further controls the reducing powers of the same samples were obtained by Bertrand's method and from the same material also the phenylosazones were isolated, their crystalline appearances examined and, when any doubt existed, the melting point recorded, alone and when mixed with an osazone prepared from pure specimens of the sugar in question. Since anaesthesia is commonly considered to cause glucosuria, as a preliminary to most experiments observations were made on the amount of reducing substances in the urine prior to the injection of diuretics. It may be said that without indused diuresis the amount of sugar or other reducing substance found in the urine as the result of ether anaesthesia in the rabbits used was so small as to be considered negligible. Urea, sodium chloride and sodium sulphate have been employed as diuretics. Of the three the last has with rabbits given by far the best results. The diuresis is rapid and sufficiently prolonged to enable an adequate quantity of urine to be collected in the arbitrary time chosen, five minutes. The urine secreted under the action of these diuretics invariably contained reducing substances, usually in small amount, and these were submitted to the same examination as the urine after injection of sugar, that is optical activity, reducing power and the isolation of the osazone. This investigation is the second part of the research and will be communicated subsequently. We do not propose to discuss this point, but we may note that the observations made show, with some certainty, that reducing substances other than glucose are excreted under the action of diuretics. Some of these substances have a lower *initial* specific rotation than that accepted in the literature for glucose, and the quantitative estimations by polarimetry and by reduction methods frequently yield conflicting results. A certain amount of evidence is in our possession to enable the conclusion to be drawn tentatively that in the rapid excretion of glucose, formed presumably by hydrolysis of glycogen, part, if not all, of the glucose excreted has a much lower optical value than either of the butylene oxide modifications. The evidence does not permit us to say that the ethylene oxide modification is formed, though the optical behaviour is significant. The definite establishment of this fact would lead to important conclusions as to the constitution of glycogen. The work is being continued.

Under the influence of concentrated sugar solutions intravenously injected temporary diuresis is produced and the administered sugar is excreted rapidly and in great part into the urine. According to Croftan [1909], but denied by Grube [1909], part is excreted into the intestine and subsequently reabsorbed into the circulation. In view of the previous work quoted above, this point may be of considerable importance. We became aware of these communications, however, after the completion of the experiments recorded below, but it is thought that, under the conditions of the present research, even if the

results of Croftan are accepted, only an inappreciable amount would have traversed this route in the time between the injection and the excretion of the sugar. Any sugar which took this route would presumably undergo stereochemical changes. We think that a complication from this possibility is remote. We assume therefore that the reducing sugar of the urines examined is composed of (*a*) hexose injected, (*b*) sugar formed from glycogen under the influence of the diuretic.

As subjects dogs and rabbits were employed, the latter in by far the larger number. The sugars investigated were *d*-glucose, *d*-fructose and *d*-galactose. The glucose and fructose were the same as used by Hewitt and Pryde in the research quoted and gave permanent optical values corresponding with those accepted as standard; the galactose was not so pure and gave in 5·6% solution $[\alpha]_D^{15^\circ} + 77^\circ$. The Bertrand solutions were standardised against pure glucose and gave results within the limits of experimental error. Copper values for fructose were taken from Bertrand's tables for invert sugar. The general technique has been indicated above. The sodium sulphate and sodium chloride were 20 % solutions in tap water and the quantity injected at a time usually was 5 cc. of the former or 10 cc. of the latter. The sugar solutions were in the cases of glucose and fructose 10 %, galactose 20 %, and were made up in tap water at least 24 hours before required to ensure that equilibrium was attained and a constant optical value reached. In rabbits 10 cc. were usually injected at once, occasionally 20 cc. Polarimetric observations were made immediately and, when a constant value was reached, the solution was treated with a trace of caustic alkali, filtered and again examined. Subsequently the phenylosazone was isolated. In view of the similarity of the results obtained with all three sugars it is proposed to give in detail only one typical experiment in which glucose was employed.

23. ix. 21. *Rabbit, male, weight* 2 *kilos, ether anaesthesia.*

Cannulae in right jugular vein and in bladder. Neck of bladder ligatured. Bladder washed out with warm 0·9 % NaCl.

11.25 a.m.	5 cc. 20 % Na_2SO_4 in tap water injected.
11.25–11.33 a.m.	Urine 5 cc. Labelled No. 1. Freely reducing.
11.33 a.m.	5 cc. 20 % Na_2SO_4 in tap water injected.
11.33–11.38 a.m.	Urine 11·5 cc. Polarimeter specimen *A*.
11.38–11.58 „	„ 14·0 cc.
12.3 p.m.	10 cc. 10 % glucose injected.
12.6 „	„ „ „
12.3–12.8 p.m.	Urine 8·3 cc. Polarimeter specimen *B*.
12.8–12.32 p.m.	„ 14·0 cc. Labelled No. 2.
12.45 p.m.	Animal died.

Analytical Notes.

Polarimeter *A*. 1 dcm. tube, sodium light, examined at once, ½ minute readings.

11.42 a.m.	0·08°	0·17°	0·17°	0·17°	0·20°	0·19°	0·20°
	0·19°	0·23°	0·23°	0·25°	0·30°	0·33°	0·27°
	0·33°	0·33°	0·30°	0·30°	0·32°	0·30°	0·32°.

Average final rotation ... ... 0·31°
Reducing material, estimated as glucose ... ... 0·59 %

After addition of a trace of alkali and filtration, mean of nine concordant readings:—

Permanent rotation ... ... 0·25°
Reducing material, estimated as glucose ... ... 0·47 %
Bertrand estimation gave concentration, estimated as glucose ... 0·36 %

Polarimeter *B*. 1 dcm. tube, sodium light, examined at once, ½ minute readings. (Readings not so sharp as in *A* above.)

12.23 p.m.	1·32°	1·28°	1·20°	1·18°	1·18°	1·20°	1·23°
	1·28°	1·28°	1·27°	1·27°	1·30°	1·30°	1·28°
	1·27°	1·28°	1·27°	1·27°	1·32°		

Average rotation ... ... 1·26°
Concentration, estimated as glucose ... ... 2·40 %

After addition of a trace of alkali, filtering and making up to twice original volume, mean of nine concordant readings:—

Permanent rotation ... ... 0·58°
Concentration, estimated as glucose in undiluted urine ... ... 2·22 %
Bertrand estimation gave concentration for undiluted urine, estimated as glucose ... ... 2·18 %.

Characteristic phenylosazones isolated from Nos. 1 and 2.

The one experiment given in detail above is typical of the results obtained generally; it shows a considerably better agreement between polarimetric and reduction estimations of "glucose" excreted under the influence of the diuretic than was sometimes found; with this proviso the figures are representative and the following can be noted:—

In *A*. (1) The slight but marked mutarotation from a low to a permanent higher optical value.
(2) A large change in observed rotation on addition of alkali.
(3) The variation in amount of reducing substance (calculated as glucose only) determined by optical and reduction methods.

In *B*. (4) The absence of any evidence of mutarotation.
(5) The small change in observed rotation on addition of alkali.
(6) The relatively good agreement between polarimetric and reduction methods of estimation.

It is probably legitimate to assume that the factors causing the variations noted in (2) are due to injection of diuretic and would thus continue to be operative in (5), and it is seen that the absolute percentage difference in (2) and (5) is, within experimental error, the same, viz. 0·12 % and 0·18 % (estimated as glucose). If this assumption is granted, it must be concluded that the glucose (or other reducing sugar) excreted in these experiments is an equilibrated mixture of the α- and β-isomerides and that no stereochemical changes of the nature found by Hewitt and Pryde have occurred. Although no explanation can as yet be given of the discrepancies in the estimations of reducing substances under the action of diuretics it can be stated that errors are not introduced by substances interfering with the accuracy of the estimations since glucose added to such urines can be recovered quantitatively. It may also be concluded that polarimetric estimation of glucose in urine, uncontrolled by other methods, may lead to fallacious results.

SUMMARY.

1. Equilibrated solutions of *d*-glucose, *d*-fructose and *d*-galactose on injection intravenously into rabbits or dogs undergo no stereochemical changes and the equilibrium is unaltered in the urine excreted.

2. Polarimetric estimation of reducing sugar in urine may give fallacious results unless controlled by other methods.

The expenses of this research have been defrayed by a grant from the Government Grant Committee of the Royal Society.

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LXXXIII. A CONTRIBUTION TO THE STUDY OF THE INTERCONVERSION OF CARBOHYDRATE AND LACTIC ACID IN MUSCLE.

By DOROTHY LILIAN FOSTER and DOROTHY MARY MOYLE.

From the Biochemical Laboratory, Cambridge. Report to the Food Investigation Board.

(*Received October 21st, 1921.*)

I. The Fate of Lactic Acid on Oxidative Recovery.

Some months ago an attempt was made to confirm some recent work by Parnas [1920] on a vexed question of the utmost theoretical interest—that of the fate, during recovery in oxygen, of the lactic acid formed in the muscle during fatigue or survival.

Until the recent series of papers by Meyerhof [1920] there have existed two views as to the explanation of the facts known concerning this recovery. The work of Fletcher and Hopkins [1907] and Parnas [1915] supplied the chemical data, and that of Hill [1911, 1914] and Peters [1913] the thermodynamic data, on which these theories were based.

The facts may briefly be stated thus: The contraction of muscle is a strictly anaerobic process, and is accompanied by the production of lactic acid. The recovery process is dependent on the presence of oxygen, and is accompanied by the removal of lactic acid.

Hill [1914] stated from calculations based on work by himself and also on that of the others mentioned above, that the heat of recovery per grm. of lactic acid removed is 450 calories, while the combustion of the same amount of lactic acid would yield about 3700 calories. From this Hill argued that the lactic acid cannot be oxidised away, but must be rebuilt into the precursor from which it arose. As the breaking down of this precursor was associated with the liberation of energy (the energy of contraction) it is obvious that a supply of energy will be needed for the reverse process. Presumably this energy is supplied by a simultaneous combustion of carbohydrate, which is responsible for the gaseous exchange during recovery.

The views held by the other school will be found in the Croonian lecture by Fletcher and Hopkins [1917]. The lactic acid was believed to be removed by being completely burnt, with the production of carbon dioxide and the consumption of oxygen, the greater part of the energy not being given out as heat, but being retained in the muscle for the restoration of the initial physico-

chemical state. Evidence for this theory was obtained by Parnas [1915], who found that more heat was given out than was found by Hill, and that the amount of oxygen absorbed was equivalent to the lactic acid which disappeared[1].

It should be noted that in the combustion of either glucose or lactic acid, the ratio of carbon dioxide evolved to oxygen absorbed is equal to unity; moreover the combustion of equivalent amounts of glucose and lactic acid yields precisely the same amount of carbon dioxide, and nearly the same quantity of heat. Hence although it has been shown [Meyerhof, 1919] that the respiratory quotient during recovery after fatigue is 1, it is quite impossible to gather from a study of the gaseous exchange and heat production alone, which of these two substances is the "fuel" of the muscle.

Parnas in a communication to the Physiological Congress at Paris [1920] describes what he considers a crucial test for distinguishing between these two hypotheses. Consider an isolated muscle whose reserve is exhausted by prolonged contractions in oxygen. Parnas states that according to the first hypothesis (that of reconstitution) the resulting non-irritable muscle should have a maximum lactic acid content, as there is no fuel present to supply the energy necessary for the rebuilding process. On the other hand, on the alternative hypothesis, the lactic acid formed will be removed by oxidation up to the last, and the muscle in its final state will contain a minimum of lactic acid.

Parnas used sartorii suspended in Ringer's solution, and stimulated at long intervals for two days. When the muscles ceased to contract, he found that the carbohydrate content had been reduced to 0·05 %, and that no lactic acid was present. No lactic acid was formed when the muscles were removed to an atmosphere of hydrogen, and under these conditions *rigor mortis* did not appear.

Believing that this argument was valid, we repeated these experiments on a larger scale and in a modified form, and, though the results have led to no definite conclusions and are in some degree incomplete, we give them in detail below as they show in a very interesting way the equilibrium between lactic acid and carbohydrate, and the extraordinary duration of irritability in amphibian muscle.

It is clear now, however, that such an experiment could not form the crucial test between the two hypotheses. Meyerhof [1920] established that on oxidative recovery one-third of the lactic acid is burnt to carbon dioxide and water, and the remaining two-thirds is rebuilt into glycogen. He showed that the oxygen absorbed is equivalent only to one-third of the lactic acid which disappears, and the heat given out during recovery is approximately equal to the heat of combustion of one-third of the lactic acid disappearing, less the heat of contraction.

It is obvious that the results obtained in Parnas' experiment are explicable just as easily on this view as on the total combustion of the lactic acid. For,

[1] Meyerhof has not been able to confirm these figures. See below.

since there is an abundant supply of oxygen, one-third of the lactic acid will always be burnt away and the energy so obtained will serve to reconstitute the precursor from the remaining two-thirds. Hence when all carbohydrate supplies are exhausted there must be a minimum of lactic acid present.

Experimental Details.

Instead of sartorii we used whole limb pairs, which were suspended in moist oxygen instead of Ringer's solution. The apparatus used was a modification of one used by Parnas [1914] for a different purpose.

The frogs used (in the two experiments described there were 60 in each case) were pithed, the hind limbs severed well above the pelvic girdle and the limb pairs immediately placed in Ringer's solution until all were ready. They were then hung by the anal apertures on aluminium hooks, which were attached to a ring of aluminium about six inches in diameter. Each ring carried thirty hooks and was provided with an aluminium stem, which passed through a hole in the rubber stopper of a large bell jar. The jar stood in a dish containing five litres of Ringer's solution, and the height of the aluminium ring was so adjusted that the limbs dipped into the solution up to the ankles. Two such bell jars were used to support the 60 frogs. An atmosphere of oxygen was maintained inside the bell jar by leading a steady, slow current of the gas from a cylinder through a glass tube which passed through the cork, and opened by means of a four-way piece below the surface of the solution. The oxygen escaped through an opening in the stopper.

The limbs were stimulated with tetanising currents, the induction coil being at 2 cms. The primary circuit contained a battery of 1·4 volts and a key. In the secondary circuit, one copper wire passed from the coil to the aluminium stem, around which it was twisted; the other wire, also of copper, was fastened to a platinum wire, carrying a platinum electrode, which dipped in the Ringer's solution. The limbs themselves completed the circuit.

The limbs were stimulated for ten minutes every hour. During the earlier periods the muscles responded actively the whole time, though signs of the onset of fatigue were visible towards the end of the periods. With the later stimulations response was obtained at the beginning of the period, but failed before the end. In two experiments the muscles remained irritable for 72 hours, *i.e.* after 720 minutes of stimulation; in the third experiment, in which midsummer frogs were used, irritability only lasted for 48 hours under these conditions. The Ringer was changed daily. Some hours before the experiment was stopped a putrefactive smell was noticed, and was believed to arise from the injured muscles of the pelvic girdle, as it is well known that muscle resists infection as long as it is excitable. At the close of the experiment the muscles were almost all non-irritable, but a few responded feebly. The difficulty of deciding the right moment for stopping the experiment is increased by this fact, that the leg muscles lose irritability at different rates.

The limbs, on removal from the apparatus, were placed in ice and shuffled. Groups of ten were selected for the various estimations, set out in detail below. In the case of those brought to heat rigor, they were placed on the bottom of thin-walled beakers, sunk in a thermostat at 45°, and kept at this temperature for 1½ hours. The lactic acid estimations were carried out exactly according to the routine method of Fletcher and Hopkins [1907] and the carbohydrate estimations by the method described by Meyerhof [1920].

Owing to individual variations in the carbohydrate content, comparable estimations were made on the *single* limbs of the same frogs whenever possible; *e.g.* for one estimation 20 left legs were used, and for the estimation to be compared with it, the 20 right legs.

Exp. 17. 13. iv. 21. (See Table I.) 60 frogs used; 72 stimulations made.

The following estimations were made:

1. Initial carbohydrate 2. Heat rigor carbohydrate	20 half limb pairs.
3. Final resting lactic acid	10 limb pairs.
4. Final resting carbohydrate 5. Heat rigor carbohydrate	20 half pairs.
6. Heat rigor lactic acid	10 limb pairs.

Exp. 18. 20. v. 21. (See Table I.) 60 frogs were used; these were very stale summer frogs, some of which had been kept a month or more, the others a shorter time, but all were very thin. Care was taken that the limbs were well shuffled. 48 stimulations were made.

The following estimations were made:

1. Initial carbohydrate 2. Final resting carbohydrate	20 half pairs.
3. Rigor carbohydrate	10 pairs.
4. Rigor lactic acid 5. Final resting carbohydrate	20 half pairs.
6. Final resting lactic acid	10 pairs.

Table I. *Exps.* 17 *and* 18.

	Initial carbohydrate (20 half limb pairs)			Final resting carbohydrate (20 half limb pairs)			Difference	Rigor carbohydrate (20 half limb pairs)			Difference	Final resting lactic acid (10 limb pairs)	Heat rigor lactic acid (20 half limb pairs)
	Glycogen	Sugar	Total	Glycogen	Sugar	Total		Glycogen	Sugar	Total			
Exp. 17	0·416	0·117	0·533	0·271	0·007	0·278	0·255	0·017	0·009	0·026	0·259	0·036	0·108
								0·016	0·003	0·019			
	(20 half limb pairs)			(20 half limb pairs)				(10 limb pairs)				(10 limb pairs)	(20 half limb pairs)
Exp. 18	0·433	0·108	0·541	0·049	0·013	0·062	0·479	0·014	0·019	0·033	0·029	0·055	
				0·037	0·015	0·052							0·032 +

Discussion of Results.

Our results agree with those of Parnas, in that the final lactic acid value was found to be a minimum. In one experiment we succeeded in reducing the total carbohydrate to the value mentioned by Parnas, about 0·05 %. On keeping the muscle at 45° for 1½ hours the carbohydrate content was reduced to 0·03 %; it was intended to estimate the lactic acid formed under the same conditions, but, owing to an accident, the result is only approximate—about 0·03 %.

In the other experiment the carbohydrate was only reduced to 0·277 %. In this case two lots of limbs were put under conditions of heat rigor, and the carbohydrates afterwards estimated; the low values of 0·026 % and 0·019 % were obtained. The rigor lactic acid in this case was 0·108 %—a high value, corresponding to the high final content of carbohydrate.

A Confirmation of some other Recent Work on this Subject.

As mentioned above, Meyerhof has recently published a series of papers in which he brings forward a great mass of evidence in favour of the reconstitution of the greater part (two-thirds to three-quarters) of the lactic acid into its carbohydrate precursor. His arguments are based on a large number of experimental results, which are different from those of Parnas, and are most convincing if these results are to be accepted. A large amount of confirmatory data is given, but all estimations were done by micro-methods, and in a matter of such fundamental importance it seemed worth while to repeat the more crucial of these experiments on a larger scale, and by different methods. Accordingly we selected two problems—the conversion of lactic acid into glycogen on recovery in oxygen and the conversion of glycogen into lactic acid on chopping—and investigated these by Fletcher and Hopkins' method for the lactic acid, Pflüger's method for glycogen and Meyerhof's modification of Parnas' method for the soluble sugars, using ten limb pairs for each estimation. We may say at once that our results were in entire agreement with those of Meyerhof.

Exp. 15. *The recovery process in fatigued muscle.*

Method. 40 limb pairs were, immediately after pithing, fatigued for 45 minutes in an atmosphere of nitrogen. The limbs were suspended from the aluminium rings as in previous experiments. A single cell of the battery was used for stimulation, and the coil was gradually pushed in as the muscle showed signs of fatigue, till finally it was at zero. After the stimulation, 20 limb pairs were removed and placed in a covered beaker sunk in ice; the whole was weighed as quickly as possible. The muscles were then dissected from one leg of each limb pair, with all the usual precautions against injury. During this time nitrogen was passed into the beaker sunk in ice. The lactic acid was extracted in the usual way. The muscles from one leg of the remaining

twenty limb pairs were then similarly treated for carbohydrate estimation. The weight of muscle was in each case determined by difference.

For the recovery, the legs with the remaining muscles were re-suspended in the bell-jars in a current of oxygen, which was maintained for 23 hours. At the end of this time the lactic acid and carbohydrate were extracted again, care being taken that corresponding legs were used, *i.e.* of the muscles of one limb pair those of one leg were used for fatigue carbohydrate and those of the other leg for the recovery value. This is necessary in view of the great individual variations in carbohydrate content.

Table II.

	Glycogen	Soluble sugars	Total carbohydrate	Lactic acid	Total carbohydrate and lactic acid
Fatigue	0·750	0·042	0·792	0·166	0·958
Recovery	0·936	0·073	1·009	0·037	1·046
Difference	...	...	0·217	0·129	

Discussion of Results.

It can be seen from these figures that, contrary to the statement of Parnas and Wagner [1914], an increase in carbohydrate content accompanies recovery in oxygen. The ratio of lactic acid lost to carbohydrate gained is, indeed, not that found by Meyerhof, but the discrepancy in our figures is no doubt to be explained by the individual variations in the limb pairs. Meyerhof, using a micro-method, was able to estimate lactic acid in the gastrocnemii of the same limb pairs used for the carbohydrate estimations.

Exp. 14. *The change of glycogen into lactic acid in chopped muscle.*

Method. The resting carbohydrate and lactic acid were determined on twenty limb pairs—twenty single legs being used for each.

60 more frogs were pithed, cooled in ice, and then skinned; the limbs were placed in beakers sunk in ice. The muscle was dissected off the limbs and chopped with cold knives, on plates resting on ice. The chopped muscle was also cooled as it accumulated. When all the muscle from the 60 frogs had been chopped, it was thoroughly mixed to obtain representative samples, and then divided into six approximately equal portions, which were weighed in tared, cooled beakers.

Table III.

Exp. 14. *The formation of lactic acid in chopped muscle.*

Lactic acid				Carbohydrate				
No.	Time	Total	Difference	No.	Soluble sugar	Glycogen	Total	Difference
5	Initial	0·044		6	0·037	0·902	0·939	
1	One hour	0·134	0·09	2	0·112	0·745	0·857	0·08
3	4 hours	0·326	0·192	4	0·158	0·527	0·685	0·172
A	Resting	0·015		B	0·05	1·18	1·23	

Four of the beakers were then sunk in a thermostat at 20°; the remaining two samples were used for the estimation of initial lactic acid and carbohydrate respectively.

The other samples were removed from the thermostat after one and four hours respectively, being placed in ice at once.

Discussion of Results.

The results obtained are again in agreement with those of Meyerhof, showing absolute parallelism between the loss of carbohydrate and increase in lactic acid, and contrary to those of Parnas, who found that the carbohydrate decrease lagged behind the lactic acid increase.

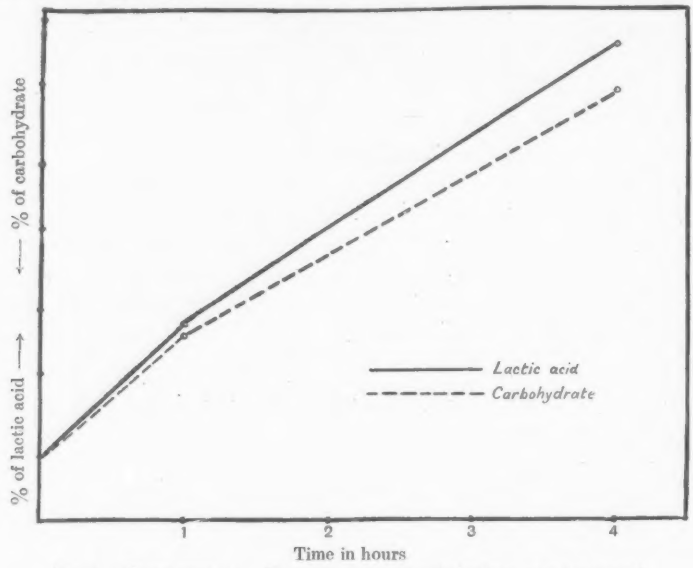

Fig. 1. Experiment 14. The conversion of carbohydrate into lactic acid in chopped muscle.

The values for the resting carbohydrate and lactic acid cannot strictly be compared with the other values, as they were obtained from distinct limb pairs. (See Table III and Fig. 1.)

Further Evidence for Hexosephosphate as the Precursor of Lactic Acid.

In this section we describe an experiment which is of some interest as it indicates that chopped muscle not only possesses the power, as shown by Embden and his co-workers [1915] for muscle juice, of breaking down added hexosephosphate to lactic acid, but that it is also capable of synthesising the hexosephosphate from added glucose and phosphoric acid.

Exp. 16. The muscle from the hind limbs of 80 frogs was dissected off and put through a mincer, being cooled the whole time in a freezing mixture. Approximately equal quantities of the mixed muscle were placed in each of four tared beakers, and weighed. Each portion was then transferred quantitatively to a 500 cc. filtrate jar, covered with a glass plate. Each lot of muscle was treated with 200 cc. of 2 % $NaHCO_3$ solution. The jars were labelled *A*, 1, 2, 3, and their contents were:—

A. Muscle + 200 cc. 2 % $NaHCO_3$ + 35 cc. H_2O.
1. Muscle + 200 cc. 2 % $NaHCO_3$ + 35 cc. hexosephosphate solution.
2. Muscle + 200 cc. 2 % $NaHCO_3$ + 35 cc. glucose solution.
3. Muscle + 200 cc. 2 % $NaHCO_3$ + 35 cc. glucose solution + 2 grms. NaH_2PO_4.

The jars were incubated at 37° for three hours, then removed and placed in a freezing mixture. The liquid in each case was strained through muslin into a beaker which was sunk in boiling water (to coagulate the protein and inactivate any enzyme) and allowed to remain there for an hour. In each case the liquid was neutralised with HCl, and to the control (*A*) 35 cc. of the hexosephosphate solution were added. The residues were extracted with ice-cold alcohol in the usual way. After standing overnight the coagula were filtered from the watery extracts, and united with those to be extracted with alcohol. The watery extracts were taken to dryness on the water bath, and the residues also extracted. The alcoholic extracts were acidified with phosphoric acid to prevent destruction of the lactic acid. From this point the routine method was followed, except that twice the usual quantity of charcoal was added. In the case of 2 and 3 the solutions were coloured, probably owing to the action of the alkali on the sugar; each of these was treated with charcoal a second time with about 1 g. and the lead carbonate treatment was also introduced. The glucose present in the moist lead hexosephosphate was estimated after hydrolysis so that the amount added was known approximately. The moist hexosephosphate was equivalent to 2·5 % glucose. A weighed amount of the Pb hexosephosphate was decomposed by H_2S, the gas removed by a current of air, and the volume made up to 80 cc. 80 cc. correspond with 11·43 g. hexosephosphate, or 0·285 g. glucose, so that 35 cc. correspond with 0·125 g. glucose. The glucose solution was made up so that 35 cc. contained 1·3 g. The results obtained are set forth in Table IV.

Discussion of Results.

These figures show conclusively that not only has the intact muscle the power of breaking down hexosephosphate *in vivo*, but that the chopped muscle apparently possesses the same power. But more interesting still is the evidence for the presence of a mechanism for synthesising this precursor from its ingredients.

Table IV.

Exp. 16. *Hexosephosphate as a precursor of lactic acid.*

No.	Treatment	Weight of muscle	Weight of zinc lactate	Weight of muscle as 100	Treatment	% of lactic acid	Difference
A.	Control	84·00	·542	100	Control	·469	
1	0·125 g. glucose added as hexose-phosphate	84·54	·742	100	0·149 g. glucose as hexose-phosphate	·652	·183
2	1·3 g. glucose added	79·781	·426	100	1·63 g. glucose	·397*	
3	1·3 g. glucose added + equiv. NaH_2PO_4	81·24	·782	100	1·63 g. glucose + equiv. NaH_2PO_4	·715	·246

* This result was unreliable owing to experimental difficulties due to the excess of glucose.

Since this experiment was performed Meyerhof [1921] has published the account of an experiment in which chopped muscle, suspended in a buffer solution containing phosphates, converted added glucose into lactic acid.

Summary.

I. Experiments are described showing the conversion of carbohydrate into lactic acid in the muscles.

II. The conversion of lactic acid into carbohydrate in the intact muscle is demonstrated, as well as the breakdown of carbohydrate in chopped muscle.

III. The breakdown and synthesis of hexosephosphate in chopped muscle are shown.

We are indebted to Professor Harden for the specimen of hexosephosphate used, and to Professor Hopkins for invaluable help and criticism throughout the work.

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LXXXIV. BLOOD ENZYMES. I. ON THE OCCURRENCE OF MALTASE IN MAMMALIAN BLOOD.

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(*Received October 24th, 1921.*)

WHILE studying the enzyme content of the blood of certain higher animals, I have frequently had occasion to observe that the ferment maltase in the blood of mammals is not a constant feature, but appears to be rather a function of the animal species. Present in the blood of one mammal, the ferment may be absent from that of another; for instance, while dog's blood serum, when tested *in vitro* under suitable experimental conditions, always shows hydrolytic activity on maltase, the serum of man, under similar conditions, I have never found to be active. As this is an observation which does not appear hitherto to have been recorded or to have attracted attention[1], I propose in the present paper to give a brief account of the experimental evidence on which it is based.

The experiments related are taken from certain general studies on blood enzymes, which have occupied me over a period of several years [Compton 1915, 1]. This explains why in what follows the experimental conditions are not uniform throughout, the activity in maltase of the different blood sera studied being determined under conditions of temperature and concentration in maltose (see accompanying Table) fixed by the main investigations in hand. From the qualitative point of view, however, in which sense the present communication is mainly put forward, these varying conditions do not in the least detract from the general results arrived at.

EXPERIMENTAL.

Blood was withdrawn as aseptically as possible by venous puncture (man, cat, dog, goat, horse, rabbit), by heart puncture (guinea-pig, rat), by bleeding at the slaughterhouse (ox, pig, sheep), and rapidly centrifuged so as to obtain a clear serum. The latter was pipetted off into a clean sterile test-tube, toluene added—one drop per cc. of serum—to ensure sterility, the tube closed with a clean sterile cork to prevent evaporation, and the mixture vigorously shaken for a few seconds. Conserved under these circumstances the serum remained as a rule sterile during subsequent weeks and months, and that whether in

[1] The idea indeed exists in the literature that in a general way maltase is to be met with in the blood, side by side with amylase and glycogenase [compare Gley 1920].

the ice-box or at room temperature: a matter of great convenience in protracted studies. The serum is referred to in the Table as "fresh" when used immediately, or at most within 2 hours of its separation from the clot; while, when used after standing a few days the age is stated. After shaking, the serum was allowed to stand a short time at the temperature of the laboratory before use, then suitable quantities were withdrawn by pipette and measured off into already prepared test-tubes containing 90 mg. of pure maltose (hydrated) dissolved in sufficient pure water to make, with the serum added, 5 cc. total solution. The tubes thus prepared had 5 drops of toluene added to each, after which they were plunged into a water thermostat regulated at a constant temperature[1]. Closed at once with clean sterile corks they were incubated for 16 hours; they were then withdrawn, uncorked, and the enzyme action stopped by heating for 6 minutes in boiling water. On cooling, the contents of each tube were diluted to 50 cc. and the proportion of maltose hydrolysed determined by Bertrand's method on 20 cc. of the diluted mixture.

The following Table gives a *résumé* of the experimental findings:

Animal	No.	Vol. of serum employed cc.	Maltose concentration of reaction mixture	Temperature of experiment ° C.	% of maltose hydrolysed in 16 hrs.	Information relating to the age of the serum when used, how it was conserved, etc.
Pig	1	0·1	*M*/20	46·0	83·8	3 days, in presence of toluene, at Lab. temp.
		0·4			92·5	
		0·8			94·3	
	1	0·02	*M*/20	46·5	28·2	8 days, Lab. temp.
		0·05			76·8	
		0·1			75·1	
		0·2			85·5	
Dog	1	0·05	*M*/20	47·0	25·4	Fresh, after centrifugation. Male, in fasting condition 48 hours
		0·1			39·1	
		0·2			50·9	
		0·4			63·5	
		0·8			78·5	
	2	0·1	*M*/20	47·0	47·9	1 day, in presence of toluene, at Lab. temp.
		0·2			68·4	
		0·4			78·5	Male, under 2 years,
		0·8			80·3	fasting 24 hours
	3	0·05	*M*/20	50·0	22·5	6 hours, in ice-chest.
		0·1			25·4	Male, state of digestion unknown
		0·2			39·1	
		0·4			52·5	
	4	0·05	*M*/20	50·0	37·5	6 hours, in ice-chest.
		0·1			57·1	Pregnant female, state
		0·2			65·2	of digestion unknown
		0·4			71·7	
	5	0·1	*M*/20	50·0	70·0	1¼ days, in ice-chest.
		0·2			82·0	Non-pregnant female,
		0·3			89·0	state of digestion un-
		0·6			96·1	known

[1] In the absence of information, at this stage, regarding the optimum temperature of maltase of animal origin, the temperature employed in these experiments has been mostly one in or around 47°, that being the optimum temperature of the vegetable maltase met with in *Aspergillus oryzae* in an action of 16 hours' duration and P_H 7·2 [Compton 1914; 1915, 2].

Table (*continued*)

Animal	No.	Vol. of serum employed cc.	Maltose concentration of reaction mixture	Temperature of experiment °C.	% of maltose hydrolysed in 16 hrs.	Information relating to the age of the serum when used, how it was conserved, etc.
Goat	1	0·06	*M*/50	42·5	11·4	1½ days, in ice-chest
Sheep	1	0·1	*M*/50	47·0	16·9	Fresh
Horse	1	0·2	*M*/20	46·0	15·5	4 days, at Lab. temp.
		0·4			24·0	
		0·8			45·0	
	2	0·1	*M*/20	47·0	11·4	6 days, at Lab. temp.
		0·2			19·7	
		0·4			35·9	
		0·8			58·7	
Ox	1	0·8	*M*/20	45·5	26·8	4 days, at Lab. temp.
Rat	1	0·12	*M*/20	50·0	4·5	11 hours, ice-chest
		0·24			16·9	
Cat	1	0·05	*M*/50	45·0	0	Fresh
	2	0·2	*M*/50	46·0	0	1 day, at Lab. temp.
Guinea-pig	1	0·25	*M*/20	40·0	0	20 hours in ice-chest in presence of chloroform
		0·5			0	
		1·0			0	
		2·0			0	
	2	0·06	*M*/50	47·0	0	6 hours, in ice-chest
Rabbit	1 and 2	0·1	*M*/20	32·0	0	Fresh
		0·4			0	
		0·8			0	
		1·5			0	
	3	0·5	*M*/20	32·0	0	Fresh
		1·0			0	
		1·5			0	
	4 and 5	0·8	*M*/20	46·5	0	1 day, at Lab. temp. Male and female ("pooled" specimen)
	6	0·06	*M*/50	47·0	0	Fresh, slightly haemolysed
	7	0·5	*M*/50	46·0	0	2 days, in ice-chest
Man	1 and 2	0·08	*M*/50	32·5	0	Fresh
	3	0·08	*M*/50	42·0	0	Fresh
	4	0·36	*M*/20	42·0	0	3 days, at Lab. temp. (positive Wassermann serum)
	5, 6, 7, 8 and 9	0·8	*M*/36	54·5	0	9 days, at Lab. temp. ("pooled" positive Wassermann sera)

It will be seen by an analysis of the foregoing Table that the enzyme maltase is present in the blood of the following animals:

Dog, Pig,
Goat, Rat,
Horse, Sheep;

while it is found to be lacking in the blood of the

Cat, Rabbit,
Guinea-pig, Man.

The great variation in enzymic activity which may be met with from dog to dog, under the influence of various modifying factors, is noteworthy. As to the relative richness in maltase of the specimens of serum from the 5 dogs, it is seen that in the case of the dogs (3), (4) and (5)—comparable together as to the temperature employed in testing—to produce in 16 hours at 50° a hydrolysis of 50 %, for instance, approximately 0·06 cc. of serum is required for dogs (4) and (5), and 0·35 cc. is required for dog (3); that is, in a general way the serum (3) may be said to be 5 times less active than either serum (4) or (5). Judged in a like manner, the serum of dog (2) is twice as rich in maltase as that of dog (1), 50 % hydrolysis being produced by 0·1 cc. of the former serum in 16 hours at 47°, while under the same experimental conditions 0·2 cc. of the latter serum is required to produce the same effect.

It is not our intention to enter here into the factors responsible for the variations observed from dog to dog, but merely to signalise the fact in passing. The question constitutes in itself a subject of sufficient interest and importance to be treated separately.

It is further to be noted that the serum of the pig is extraordinarily rich in the ferment maltase. To produce a 50 % hydrolysis of maltose under the experimental conditions here involved, only 0·03 cc. of serum is required, a dose considerably less than that for any of the other animals examined. This outstanding comparative richness of pig's serum in maltase was first pointed out by Doxiades [1911], although the presence of the enzyme in the blood serum of the pig has been known since the work of Tebb [1894].

Consider now the animals in the preceding Table in whose blood the enzyme has failed to be revealed: cat, guinea-pig, rabbit and man. It appeared to us necessary, before definitely concluding the ferment to be absent from the blood of these mammals, to investigate:

(1) Whether the enzyme might not be retained in the clot and cellular elements during the process of coagulation and ultimate separation of the serum; in which case by provoking a plasmolysis of the cells the enzyme might reasonably be expected to be liberated, if it were present, and thereby become evident.

(2) Whether the enzyme might not be present in some masked or latent state, such as the alkalinity of the serum might possibly produce; in which case one would expect the enzyme to be liberated, or activated, by acid neutralisation.

To test (1), the following experiments were undertaken with the blood of the guinea-pig and the rabbit:

(*a*) *Guinea-pig.* A guinea-pig was etherised, and after having the jugular cut, was bled into a dry clean centrifuge tube. The blood, on centrifugation, yielded about 6 cc. of serum, which was pipetted off as completely as possible from the clot. To the latter were added 6 cc. of pure water, and about 20 drops of chloroform, and, after mixing, it was set aside in the ice-chest for 24 hours; then centrifuged and the resulting supernatant dark red solution examined for

activity on maltose. Quantities of the haemolysed solution ranging from 0·25 cc. to 1·5 cc. were added to tubes containing 90 mg. of maltose and water to 5 cc. and the tubes incubated at 39° for 16 hours. No activity in maltase was exhibited under these conditions.

(*b*) *Rabbit.* Blood was withdrawn from the ear-vein, and centrifuged immediately to separate serum from clot. To the latter, after decanting off the serum, was added a volume of chloroform water equal to that of the serum removed, and after thorough admixture it was allowed to macerate overnight in the ice-chest. The haemolysed liquid of maceration was tested in quantities ranging from 0·5 to 1·0 cc. for activity in maltase, and was found to be quite inactive.

These two experiments show that maltase failed to be extracted from the blood cells of the clot, and, one is justified in assuming its absence as such.

To test (2), a pooled specimen of fresh human serum from three healthy persons was added in volumes of 0·15 cc. to test-tubes containing 90 mg. of maltose dissolved in 5 cc. of pure water which had previously been acidified by the addition of progressive volumes of N/H_2SO_4: 0·1, 0·3, 0·6 cc.; and after 16 hours' incubation at 36°, a determination of the maltose hydrolysed was made by Bertrand's method. No hydrolysis was found to have occurred in any of the tubes[1].

This experiment seems to show that if a serum in the natural state does not indicate the presence of maltase, the enzyme may be taken as absent, since alteration of hydrogen ion concentration fails to bring it into evidence.

Conclusion.

Mammals can, therefore, be divided into two groups: according to whether they have, or have not, maltase in their blood. To the former group belongs the dog, to the latter the rabbit; the two laboratory animals which have hitherto been mostly used in connection with the experimental study of sugar transformations in the blood. The fact that man belongs to the non-maltase or "rabbit group" makes the observation of rather more than passing interest: and that whether viewed from the physiological, the pathological, or the medico-legal point of view.

The conception is illuminating in the explanation it affords of various contradictory statements met with in the literature. Thus, when it is stated—as the result of injecting maltose into the blood stream and studying its elimination by the urine—that the sugar of the blood cannot be maltose [see footnote p. 681], it is obvious the animal worked with must have belonged to the maltase or "dog group." In effect, this is so; for Dastre and Bourquelot [1884],

[1] In this connection it may be stated that according to certain of our experiments, to form the object of a subsequent communication, by alteration of the hydrogen ion concentration of dog's serum—which as we have seen is normally rich in maltase—by the addition of 0·15 cc. $N/100$ H_2SO_4 to a reaction mixture similar to the above, containing 0·1 cc. of serum, the activity of the maltase present is increased by 15% in an action of 16 hours' duration at 50°.

on whose work this statement is probably founded, working with the dog, found that most of the maltose injected was not voided by the kidneys but was utilised, with much the same facility as glucose. On the other hand, Philips [1881] is reported by Green [1899] to have found that when maltose is injected into the blood of an animal—species not stated, and I have been unable to trace the original publication—it is mostly excreted unchanged in the urine, much as the cane-sugar in a certain famous experiment on carbohydrate assimilation of Claude Bernard [1849]. There appears to me little doubt that in this case the animal worked with belonged to the non-maltase group.

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LXXXV. UROCHROME AS A DERIVATIVE OF CHLOROPHYLL.

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In a series of papers Palmer and his co-workers have shown that many animal pigments are derived from carotene and xanthophyll [Palmer and Eckles 1914; Palmer and Kempster 1919], therefore it was thought desirable to find out if urochrome also is derived from plant pigments.

Preliminary experiments were performed with human urine. The urine was collected in twenty-four hour samples, preserved with chloroform and the urochrome was extracted by the method of Garrod [1874] from one tenth of each daily quantity and the alcohol extracts were compared by a colorimeter.

By restricting the amount of plant pigments in the diet the colour extracted by the above method was reduced by about one fifth. This led to experiments being carried out using guinea-pigs, as it is possible to give them diets with greater variation in plant pigments. The urochrome was extracted as above by Garrod's method and the amount of colour was estimated from the volume of the extract and its depth of colour.

The experiments show that a diet deficient in plant pigments is associated with a urine deficient in yellow pigment. As the chemical composition of urochrome indicates that it is an acid substance giving reactions for pyrrole [Dombrowski, 1907], and might be a derivative of chlorophyll [Willstätter and Stoll, 1913], further experiments were carried out contrasting a diet containing carrots with one containing green pigments (carrot tops) with the result that the green parts of plants caused a marked increase in the amount of colour in the urine whilst the yellow pigments did not show any appreciable effects.

The experiments are so easy of repetition that full experimental details need not be given. One experiment on a guinea-pig is included to show the extent of variations in colour produced by changes in diet.

Guinea-pig weight 335 g.

Date 1921	Diet	Volume of urine c.c.	Relative colour of extract
30. v.	Bread and greens	discarded	
31. v.–2. vi.	„ „	69	127
2–4. vi.	„ „	72	216
4–6. vi.	Bread and apple	discarded	
6–8. vi.	„ „	32	60
8–10. vi.	„ „	45	41
11–13. vi.	Bread and carrots	discarded	
13–15. vi.	„ „	53	48
15–17. vi.	„ „	95	39

This experiment shows the striking effect of greens in the diet on the colour of the urine. The averages are: diet containing carotene 44, diet without obvious pigments 51, diet with chlorophyll in addition to carotene 172.

If urochrome is a derivative of chlorophyll one ought to expect to obtain similar pigments, for instance during digestion, from parts of plants which contain chlorophyll. Pigments agreeing with urochrome in colour, in absence of absorption bands in the visible portion of the spectrum and in solubilities have been obtained by direct extraction from hay and tea leaves; and from cabbage and spinach on treatment with dilute acid followed by alkali.

Pelkan [1920] states that urochrome is related to the protein in the diet. His results may be due to plant derivatives associated with the proteins used. In this connection it should be noted that the colour of milk whey is due to a pigment similar to urochrome. [Palmer and Cooledge, 1914.]

SUMMARY.

Green parts of plants increase the output of urochrome in the urine whereas carotene does not. In view of the chemical similarity of chlorophyll derivatives and urochrome it seems probable that urochrome is a derivative of chlorophyll.

The apparatus for this research was obtained by means of a grant from the London Hospital Medical College Research Fund.

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LXXXVI. THE ESTIMATION OF CARNOSINE IN MUSCLE EXTRACT.
(PRELIMINARY NOTE)

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With a view to the study of the physiological significance of carnosine, it was first necessary to devise a method of estimation applicable to small quantities of material, which, at the same time, would give more accurate results than have hitherto been obtained with this substance [*vide* von Fürth and Hryntschak, 1914]. A general method of estimation of iminazole derivatives by a modification of the diazo reaction described by Koessler and Hanke [1919]—but not applied by those workers to carnosine—has here been tried as a likely means of affording a reliable indication of the carnosine content of muscle[1]. Before the method could be satisfactorily tested, it was first necessary to prepare pure carnosine and for comparative purposes also histidine.

Histidine monohydrochloride was prepared from ox-blood by the method of Fränkel [1907]. M.PT. 253°, with softening and loss of water at 175–180°.

Kjeldahl. Nitrogen. Found 19·79 %. Calculated 20·05 %.

Preparation of Carnosine. This was obtained from Lemco. The most successful method found for the preparation was that adopted by Baumann and Ingwaldsen [1918] with the use of mercury acetate followed by the usual silver-baryta precipitation. A very readily crystallisable product is obtained if, after the addition of excess silver nitrate solution and before filtration of the purine fraction, barium hydroxide solution is added to more than neutralise the nitric acid set free by the use of the silver nitrate reagent. Otherwise, part of the purine group remains dissolved and escapes precipitation. This incomplete separation of the purine fraction from the carnosine fraction probably accounts for much of the difficulty workers have experienced in the isolation of pure carnosine [*vide* Gulewitsch, 1913; Smorodinez, 1913]. On first attempting the preparation this difficulty was met with. On precipitating the carnosine fraction, which refused to crystallise from alcohol, with the above precaution the product was readily purified. The purine part which

[1] Clifford [1921], since the commencement of this research, has applied a modified Koessler and Hanke procedure to the estimation of carnosine. As to both treatment of material and results found, the writer is so much at variance with the above worker that a publication at the present stage of this research has been deemed desirable.

separated here was treated with hydrogen sulphide, concentrated, and a syrup separated on treatment with alcohol. This appears at least presumptive evidence for the validity of the foregoing suggestion of the cause of the difficulty. The substance was first isolated as carnosine nitrate. This agreed in properties with those described by Gulewitsch and others [*vide* Gulewitsch and Amiradžibi, 1900; Gulewitsch, 1913]. M.PT. 220°.

The free base was prepared from part of the above product by reprecipitation with silver nitrate and barium hydroxide, and concentrated without neutralisation. The carnosine thus obtained was similar to that of Gulewitsch; M.PT. 260° with decomposition.

Part of the carnosine was used to prepare the copper-salt according to the directions of Mauthner [*vide* von Fürth and Hryntschak, 1914]. Deep-blue hexagonal crystals about 1 mm. in greatest length were obtained.

Solutions of 0·1 % carnosine were made from the free base and from the carnosine nitrate after these had been dried in desiccator to constant weight. Equal quantities of these tested colorimetrically agreed almost perfectly.

It was further intended to make a standard solution from the copper salt but the difficulty of getting this to constant weight, along with the rapidity with which it took up moisture when dry, led to the acceptance of the value as confirmed by the free base and its nitrate.

The Colorimetric Method.

Working mainly from certain theoretical considerations of the properties of diazonium compounds and their mode of coupling, Koessler and Hanke have, undoubtedly, greatly enhanced the value of the diazo reaction as a method of estimating iminazole derivatives. The use of a relatively stable colour-standard is only a small part of the improvement they have effected on the previously somewhat haphazard way of performing the diazo test.

As a quantitative method for the estimation of histidine the diazo reaction had been used by Weisz and Ssobolew [1914] and their method was adopted for the estimation of carnosine in mammalian muscle by von Fürth and Hryntschak. The much more consistent results obtainable by the Koessler and Hanke procedure testify to its superiority over any previous method.

The method has been adopted here almost *in toto* for the estimation of carnosine and found to give very satisfactory results in pure solutions of that substance. Different proportions of methyl-orange and Congo red had of course to be employed to get a match for the carnosine solutions. A very suitable match for pure carnosine solutions was obtained with 0·10 cc. methyl-orange and 0·25 cc. Congo red per 100 cc. water. These amounts were taken from 0·1 % stock methyl-orange and from 0·5 % stock Congo red.

Molecular colour values. To test the purity of the above-mentioned histidine compound and at the same time to verify the accuracy of the method, the molecular colour value of histidine was determined according to the (CR-MO)

colour-standard employed by Koessler and Hanke. This was found to be approximately 117,000,000 mm. as compared with 114,000,000 mm. found by those workers. The slight discrepancy between these results can be accounted for by experimental error, along with the possibility that there might be slight differences in the quality of the methyl-orange and Congo red employed.

The molecular colour value of carnosine was found to be 152,000,000 mm. against the same indicator.

Question as to the linearity of colour production. Clifford [1921] questions the direct proportionality between colour-production and the amount of iminazole in solution. The linear relationship, as set forth in the ideal tables of Koessler and Hanke, was tested for both histidine and carnosine by the writer and accepted within the limits of experimental error before the publication of the paper above referred to. It was very carefully retested on Clifford's figures coming to his notice, but the conclusion reached was merely a re-confirmation of Koessler and Hanke's results. Histidine was tested only within the limits of 0·01 mg. and 0·05 mg. histidine monohydrochloride in the test cylinder, whilst carnosine was tested between 0·005 mg. and 0·2 mg. The following table shows the results for carnosine, using a difference of 0·005 mg. carnosine between each reading up to 0·05 mg.

Carnosine in test cylinder mg.											
0·005	0·010	0·015	0·020	0·025	0·030	0·035	0·040	0·045	0·050	0·1	0·2
Reading of standard cylinder mm.											
3·2	5·7	8·9	11·8	14·8	17·5	20·8	23·8	26·9	30·0	59·7	120
Calculated reading of standard cylinder mm.											
3	6	9	12	15	18	21	24	27	30	60	120

When 0·1 mg. and 0·2 mg. carnosine were present in the test cylinder, the plunger was set at 10 mm. and 5 mm. respectively, and the reading of the standard cylinder multiplied by 2 or 4 gave the value equivalent to the test cylinder set at 20 mm.

The results for histidine within the limits tested were similar in their linearity.

Clifford finds colour values greater than those given by calculation on the linearity basis, in the more concentrated iminazole solutions. Exactly the opposite results were found by the writer, when the usual concentration of diazonium sulphonate reagent was used for widely differing concentrations of carnosine. Experiments here showed that the time required for maximum colour development increased with the amount of carnosine present so long as that maximum was the theoretical value. This was especially marked when more than 0·045 mg. carnosine was used. To give a few practical examples, it may be noted that with 0·045 mg. the theoretical value was reached only after standing for 12 to 14 minutes; with 0·05 mg. it was almost reached after 20 minutes; with 0·1 mg. and 0·2 mg. the highest readings were obtained only after 15 and 10 minutes respectively, but both of these were short of the

theoretical. With 0·1 mg. this shortage was 7 mm., *i.e.* approximately 12 %. With 0·2 mg. the reading was 24 mm. low, *i.e.* 20 %. If, however, a more concentrated diazonium reagent is used, the time necessary for maximum colour development is decreased, and the actual maximum is then approximately theoretical as shown in the foregoing table.

These results are quite in accordance with the mass action law. In presence of a relatively large amount of carnosine the diazonium sulphonate is in great measure used up, with the result that the speed of coupling under these conditions becomes low, and at a certain point practically nil. All the diazonium compound is not, however, used up with 0·1 mg. carnosine as the behaviour with 0·2 mg. shows. Here, the presence of a higher concentration of carnosine accounts for a greater amount of coupling, yet a similar equilibrium becomes established before complete coupling of the carnosine has taken place.

These facts merely indicate that a definite excess of diazonium sulphonate is necessary to complete the chemical reaction in one direction. They do not at all affect the validity of the linear relationship.

It is difficult to account for the deviation found by Clifford.

The best results were obtained when the test cylinder contained 0·02 mg.–0·04 mg. carnosine. The maximum is reached in 8–12 minutes after mixing, and is stable at those concentrations for approximately another 8 minutes.

No appreciable colour was developed by the interaction of the sodium carbonate used (Merck's) with the diazo reagent alone, even after 20 minutes.

Certain Considerations preliminary to the Estimation of Carnosine in Muscle Extract.

Filter paper adsorbs carnosine. Koessler and Hanke [1919] show that iminazoles are adsorbed by animal charcoal. This fact suggested that the use of filter paper might vitiate the results in such a microchemical method as that here employed. The adsorptive effect of filter paper was shown in the following way.

20 cc. of a cold solution of carnosine with a colour value of 13·9 mm. per cc. were run from a pipette through a 15 cm. diam. dry filter paper into a dry measuring cylinder. 1 cc. of this filtrate was then tested and found to have a colour value of only 11·5 mm. This process was thrice repeated, through three papers, and 1 cc. of the filtrate gave successively colour values of 10·0 mm., 8·5 mm., and 7·7 mm. That is, the total loss in colour value was 6·2 mm. per cc., or approximately 44 %.

In order to determine to what extent this loss was recoverable by washing, 10 cc. solution of carnosine with colour value of 18·0 mm. per cc. were added to four dry filter papers as above but folded into one funnel. The results are expressed in the following table:

Added to filter	Volume of filtrate cc.	Colour value of filtrate per cc. mm.	Total colour value of filtrate mm.
10 cc. carnosine solution	3·2	8·5	27·2
10 cc. water	9·2	5·5	50·6
20 cc. „	19·8	2·5	49·5
40 cc. „	evaporated to 27·0	1·0	27·0

The loss as shown in the first filtrate is 9·5 mm. per cc., *i.e.* approximately 53 %. The sum total of colour values of filtrates is 154·3 mm. The total colour value of the original solution is 180 mm. so that, after washing with water to the extent of seven times the volume of the original carnosine solution, there yet remains 14 % of the original carnosine held by the filter paper.

For these reasons, glass wool, which shows no adsorptive properties, has been adopted for filtration purposes.

The effect of heat on the colour value of pure carnosine solutions. Several clean, dry duroglass test-tubes were constricted to a neck of about 3 mm. diameter about 3 cm. from the bottom. A horizontal mark was made with a file at the constriction. (The object of constricting the tubes was to obtain more accurate measurement.) The tubes were filled to the mark with solutions of carnosine of known colour value and set on a boiling water-bath for 1 hour. They were then removed, cooled, and made up to the mark with water. A number of solutions of carnosine with an original colour value of 19·5 mm. per cc. showed after this treatment an average loss in colour value of 1 mm. per cc. Similarly a number of solutions of carnosine nitrate with an original colour value of 18·5 mm. per cc. showed after the same treatment an average loss of 0·5 mm. No change of tint in the colour developed was observable in these tests.

It is probable from these results that a process of extraction which involves prolonged boiling may be harmful to the carnosine.

Method of Extraction employed.

The most consistent results have been obtained by the following procedure. A small quantity of muscle, say 1·5 g., is extracted with about 20 cc. water on a bath at 70°. After 30 minutes the solution is filtered through glass wool into a small Buchner flask. The meat residue is transferred to a mortar, and ground with glass to a fine pulp. This is again extracted with 20 cc. water for 15 minutes, poured through the same filter and sucked as dry as possible. The top layer of wool and meat residue is re-transferred to beaker, stirred up with a further 20 cc. water and heated another 15 minutes. It is then re-filtered and washed twice with 10 cc. warm water, sucking as dry as possible between washings. After this treatment the final filtrate should give no diazo reaction.

One extraction, found by Clifford [1921] to be sufficient, has never given satisfactory results in the writer's hands. After extracting the finely ground tissue once with a large volume of water, followed by copious washing, the

colour value has shown a loss of 6 to 10 % reckoned as carnosine, as compared with that from the same meat extracted as directed above.

The total milky filtrate—representing about 50 cc. per g. of meat used—is poured into a suitable beaker. The reaction is made slightly acid with dilute acetic acid and the beaker set on the water-bath at 70° for a few minutes. Under these conditions the proteins are very effectively precipitated. The coagulum settles quickly and is readily caught in the glass wool filter. The contents of the filter are washed thrice with a little warm water. The final filtrate should be quite clear and entirely negative to all protein tests.

The results are practically unaltered if instead of the above method excess of lead acetate followed by excess of disodium phosphate is used to precipitate the protein. Thus three samples from a piece of lean muscle, extracted as above, showed on reckoning the total colour value as carnosine 0·355 %, 0·369 % and 0·380 %. In the first two samples the proteins were precipitated with lead acetate and sodium phosphate; in the last by the acetic acid method. The acetic acid method was finally adopted because it is more simple and gives a smaller precipitate by which less carnosine is likely to be adsorbed.

Further it would appear that there are no substances in muscle extract as prepared above which inhibit the diazo colour production; thus 1 cc. meat extract with colour value 22·0 mm. per cc. was mixed with 1 cc. carnosine solution 18 mm. per cc., and 1 cc. of the mixture tested. This was found to have a colour value of 20 mm. which is the theoretical number.

The total colour value of muscle extract as obtained by the methods outlined above, is consistent for the same material to within 3–4 % of the colour value reckoned as carnosine in meat. Thus three pieces of ox muscle yielded 0·388 %, 0·378 % and 0·379 %. Another sample of ox muscle gave 0·614 %, 0·606 % and 0·620 %. These materials were purchased from a butcher. A cat muscle in three portions showed 0·280 %, 0·287 % and 0·293 %.

As yet, however, the method does not appear to be entirely satisfactory for the estimation of carnosine in muscle extract. Carnosine is, undoubtedly, responsible for the production of the great bulk of the colour in these muscle extracts, but the writer has good reason to believe that certain other substances contribute to a considerable degree to the yielding of the total diazo value. Further investigation is in progress on this point.

My thanks are due to Prof. Cathcart for suggesting this research, and for his help throughout it. The expenses were in part defrayed by a grant made by the Carnegie Trust to Prof. Cathcart.

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LXXXVII. A STUDY OF THE COMBINED ACTION OF RAW COW'S MILK AND ORANGE-JUICE AS ANTISCORBUTIC SUBSTANCES.

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Introductory.

The experiments to be described were devised to determine whether raw cow's milk can replace orange-juice quantitatively as an antiscorbutic substance. If this proved to be the case it was proposed to utilise the results to estimate the antiscorbutic value of human milk.

It has been shown by Chick, Hume and Skelton [1918, 1] that raw cow's milk has a low antiscorbutic value. In the case of guinea-pigs, when less than 50 cc. were consumed daily, the animals showed no protection from scurvy. If 50–85 cc. were taken weight was better maintained. When 100–150 cc. formed the daily ration the animals grew normally and showed no symptoms of scurvy.

When such large quantities of milk have to be administered daily, the conditions of the experiment become difficult, as unless the animal co-operates heartily, it is impossible to obtain a daily consumption of more than about 80 cc.

It follows that if human milk has a comparable antiscorbutic potency, its direct quantitative estimation would not be easy to carry out.

Orange-juice, on the other hand, is very rich in antiscorbutic substance. Davey [1921] has carried out careful experiments to determine within narrow limits what is the minimum ration which will prevent scurvy. It was found that the animals receiving only 0·5 cc. of orange-juice developed scurvy. Of eleven animals receiving 1·5 cc., six were protected, three died of intercurrent disease, while only two succumbed to scurvy. The minimum ration which will allow of normal life is therefore 1·5 cc., though a certain proportion of animals employed will not show complete protection when receiving this amount.

If raw cow's milk and orange-juice could replace one another in direct proportion to their respective antiscorbutic values, by using both these substances at one time as the source of the antiscorbutic factor, much smaller quantities of milk would have to be administered. The conditions of the experiment would be facilitated, and by carrying out parallel experiments with human and cow's milk, it would be possible to obtain comparable values for cow's milk and human milk in terms of orange-juice.

The ration of cow's milk to be employed had first to be decided on. It was thought most convenient to administer about one-quarter of the minimum milk ration, *i.e.* about 35 cc. daily. It will be shown in the subsequent discussion that this ration has an adequate amount of the fat-soluble vitamin and will permit of quite satisfactory growth, though it is less than the amount necessary for optimum development.

The orange-juice ration was likewise divided into quarters, *i.e.* 0·5 cc., 1·0 cc., 1·5 cc., 2·0 cc., etc., and combinations of 35 cc. of cow's milk with varying amounts of orange-juice were tested on a series of animals.

Technique.

Young guinea-pigs of about 250–350 g. were employed. The basal ration consisted of oats and bran moistened and rubbed together, and water *ad lib.* Measured amounts were presented daily and the amount actually consumed was carefully noted. The animals were divided into three groups.

A. The first group received 40 g. of cabbage in addition to the basal ration and thus served as normal controls.

B. The animals in the second group received 35 cc. of milk which had been heated to 120° for one hour in the autoclave. In addition, varying amounts of orange-juice from 0·5 cc.–2·5 cc. were given (see Table I).

C. The third group received 35 cc. of raw cow's milk and varying amounts of orange-juice (see Table II).

Table I.

Animal	Addition of orange-juice to basal diet + 35 cc. autoclaved milk cc.	Duration of expt. (days)	Remarks	Post-mortem
B 1	0·5	72	Left hind limb raised off ground and tender.	Haemorrhages. Widening of costochondral junctions. Left knee joint ankylosed.
B 2	1	68	Ankylosis right hind limb.	Haemorrhages. Costal junctions blurred. Lymph glands congested.
B 3	1·5	68	—	Normal.
B 4	2	54	—	Normal.
B 5	2·5	52	—	Normal.

Table II.

Animal	Addition of orange-juice to basal diet + 35 cc. raw milk cc.	Length of expt. (days)	Remarks
C 1	0·5	76	No symptoms of scurvy. Nil abnormal post-mortem.
C 2	1	60	
C 3	1·5	54	
C 4	2	56	
C 5	2·5	50	

The results obtained are summarised in the accompanying tables and diagram (Tables I and II and Fig. 1).

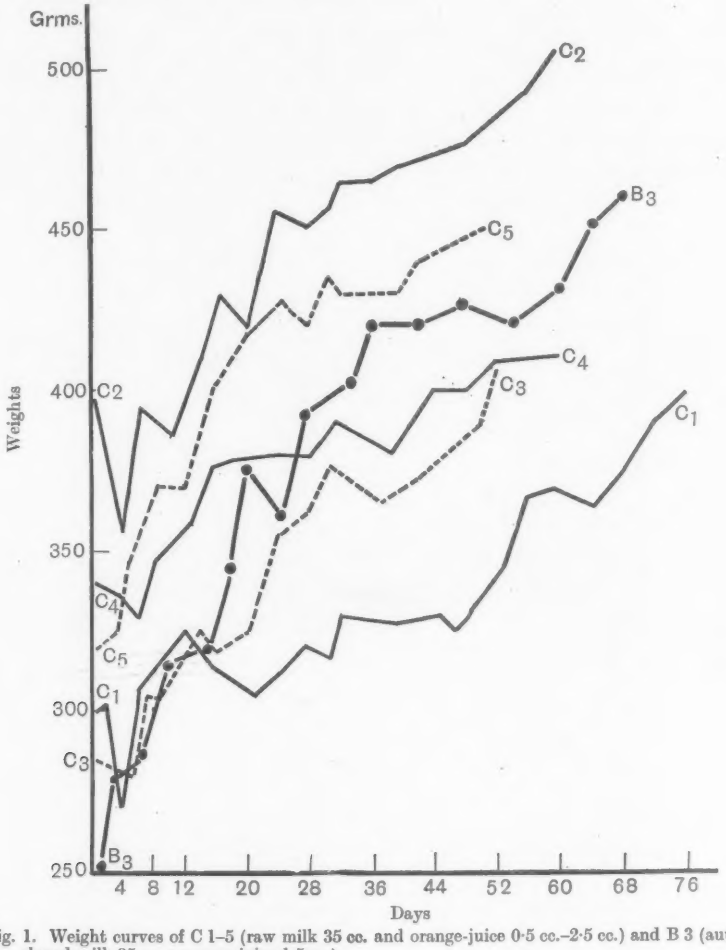

Fig. 1. Weight curves of C 1–5 (raw milk 35 cc. and orange-juice 0·5 cc.–2·5 cc.) and B 3 (autoclaved milk 35 cc. + orange-juice 1·5 cc.).

Discussion.

It was thus found that of the animals fed on autoclaved milk only those receiving 0·5 cc. and 1·0 cc. orange-juice (B 1 and B 2) developed scurvy. B 3, which received 1·5 cc. daily, showed no abnormal signs. B 4 and B 5, receiving 2·0 cc. and 2·5 cc. of orange-juice daily, developed quite normally and no suggestive signs were found on post-mortem examination. These results are in agreement with those of Davey [1921] already quoted.

None of the guinea-pigs in the series C receiving raw milk showed any signs of scurvy during life, or any characteristic changes post-mortem. It thus appears that 0·5 cc. of orange-juice when combined with 35 cc. of raw milk was sufficient to prevent the onset of the disease.

A second confirmatory set of experiments was then performed. The basal ration consisted of oats and bran and water *ad lib.* 0·5 cc. of juice was administered to all the animals. In addition, small quantities of raw cow's milk, varying from 15 cc.–40 cc. were given. In the case of animals receiving less than 40 cc. of raw cow's milk, their total milk ration was made up to 40 cc. with autoclaved milk.

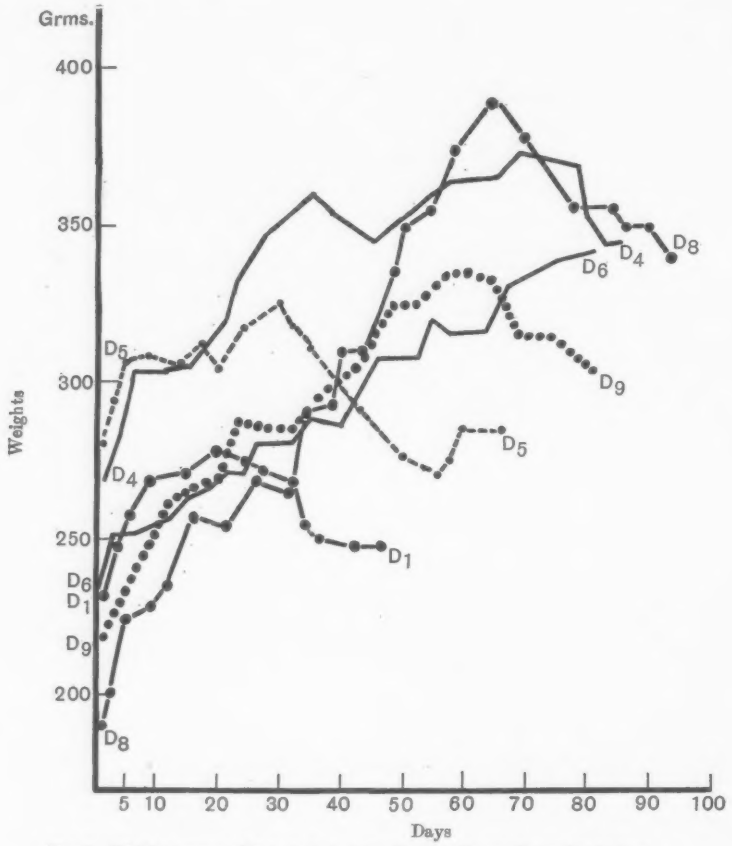

Fig. 2. Weight curves of D 1, 4, 5, 6, 8, 9. For details see discussion in text.

The results obtained are summarised in the accompanying table and diagram (Table III and Fig. 2).

Table III.

Animal	Addition of milk to basal ration + orange-juice 0·5 cc.	Length of expt. (days)	Remarks	Post-mortem
D 1	Raw milk 0 cc. Autoclaved milk 40 cc.	46	Face-ache position very definite	Emaciated. Definite haemorrhages into muscles. Costo-chondral junctions thickened. Lungs and intestines normal (scurvy present)
D 2	Raw milk 0 cc. Autoclaved milk 40 cc.	39	Tender limbs, looking ill (died)	Emaciated. Pneumonia. Haemorrhages. Thickened and congested costo-chondral junctions (scurvy present)
D 3	Raw milk 15 cc. Autoclaved milk 25 cc.	46	Lies crouched up. Looks ill	Emaciated. Intense congestion of costo-chondral junctions. Gastro-enteritis and Pneumonia (scurvy present)
D 4	Raw milk 15 cc. Autoclaved milk 25 cc.	86	Fit	Costo-chondral junctions normal. Intestines normal (no scurvy)
D 5	Raw milk 25 cc. Autoclaved milk 15 cc.	66	Limping slightly. Thin	Well-marked haemorrhages. Intestine healthy. Slight pneumonia. Costo-chondral junctions obscure. Two or three ribs matted together. Spontaneous fracture of rib and excessive callus formation (scurvy present)
D 6	Raw milk 25 cc. Autoclaved milk 15 cc.	81	Normal	Normal (no scurvy)
D 7	Died suddenly while being hand-fed			
D 8	Raw milk 35 cc. Autoclaved milk 5 cc.	93	Condition good. Appears healthy	Intestine and lungs healthy. Slight haemorrhages. Most of rib junctions on both sides show marked deformity from chronic overgrowth. Appearance resembles "rickety rosary" (? scurvy present)
D 9	Raw milk 35 cc. Autoclaved milk 5 cc.	81	No symptoms of scurvy. Not looking well	Lungs and intestine healthy. Nil abnormal found (no scurvy)
D 10	Raw milk 40 cc. Autoclaved milk 0 cc.	60	Normal	Nil abnormal found (no scurvy)

D 1 and D 2, which received no raw milk at all, developed definite symptoms of scurvy.

In the case of the remaining animals, D 3–D 10, owing to the general vagueness of the signs shown during life, a definite diagnosis could only be arrived at after a careful consideration of the weight charts taken in conjunction with the findings after death. Great stress was laid on the presence or absence of haemorrhages, but due attention was also paid to the bony changes. Histological bone lesions were not taken as evidence of scurvy in the absence of haemorrhages, as Tozer [Delf and Tozer, 1918] has shown that such lesions frequently occur in animals on non-scorbutic diets, and are probably due to deficiency of vitamin A.

Since the early days of the work on scurvy, it has been known that the weight curves did not vary solely with the antiscorbutic substances present in the diet. Chick, Hume and Skelton [1918] found it necessary to administer 60 cc. of autoclaved milk daily to obtain normal growth curves in the case of guinea-pigs which were receiving an adequate ration of the antiscorbutic substance. Hume [1921] has recently made an extensive investigation into the power of various natural foodstuffs to promote growth in guinea-pigs. In the case of cow's milk, only a limited number of experiments were performed. Guinea-pigs receiving 60 cc. of raw cow's milk daily showed an average gain of 46·9 % in weight, which closely approximates to the increase in the controls of 55·8 %. Two animals were also placed on 20 cc. of raw milk daily. At the end of 25 days one showed a slight loss and the other a slight increase in weight. After 30 days both were put on 40 cc. daily and subsequently showed a slow recovery.

In the case of autoclaved milk, 60 cc. daily resulted in an increase of 44·8 %.

On analysis of the results obtained in the first series of experiments (Table IV), it is found that the average increase in weight obtained at the end of 45 days was 33·5 %. This may be compared with the results of Hume and shows that 35 cc. of cow's milk daily contains appreciably less than the optimum amount of vitamin A. Quite good growth, however, occurs on such a diet.

Table IV.

Animal	Original weight	After 45 days	% increase
C 1	299	320	7·5
C 2	398	475	19·6
C 3	380	285	33·7
C 4	390	340	14·7
C 5	445	326	36·5
B 3	420	238	76·5
B 4	380	258	47·3
B 5	380	230	65·4

Average: 33·5 %

It is now possible to discuss the history of each of the animals receiving raw milk. From examination of the details given in Table III, it will be seen that D 3 and D 5 alone (receiving 15 cc. and 25 cc. of raw milk respectively) developed definite signs of scurvy. D 4, D 6, D 9 and D 10 all grew well, putting on from 50–100 % in weight, and no signs of scurvy could be detected.

D 8 maintained very steady progress for 65 days, putting on over 100 % in weight. During the last month of the experiment he fell off a little. He was killed on the 93rd day when the condition was quite good. As stated in the protocols, a definite diagnosis of scurvy could not be made.

Taking the above facts into consideration, the experimental findings may be summarised thus:

Definite scurvy	D 3	(15 cc. raw milk)
	D 5	(25 cc. ,,)
Doubtful scurvy	D 8	(35 cc. ,,)
No scurvy	D 4	(15 cc. ,,)
	D 6	(25 cc. ,,)
	D 9	(35 cc. ,,)
	D 10	(40 cc. ,,)

It is, therefore, possible to conclude that, when such small quantities of raw cow's milk as 15–35 cc. are added to a basal ration already containing 0·5 cc. of orange-juice, protection from scurvy may be obtained. This figure is considerably less than what one would expect if raw cow's milk and orange-juice interchanged in direct proportion to their respective antiscorbutic values. On a quantitative basis the ration of antiscorbutic substance administered amounted to half or less than half the minimum requirements of the animals. It thus appears that when a mixture of antiscorbutic substances are administered, the combination is somewhat more potent than when proportionate amounts of the same substances are administered separately.

It must be remembered, however, that different samples of cow's milk may show considerable variation in their antiscorbutic content. No direct determination was made of the minimum dose of the milk employed, the figure obtained by Chick, Hume and Skelton [1918] being adopted. Any such determination is liable to a probable error of 25–50 %. In view of these facts the results obtained need not necessarily involve any very marked difference between the combined and separate actions of cow's milk and orange-juice.

The opportunity was taken during the course of the present research to investigate the condition of the lymphoid tissue in scurvy. Cramer, Drew and Mottram [1921, 1] have shown that in rats fed on diets deficient in vitamins A and B, the only constant and characteristic change found is marked hypoplasia of the lymphoid tissues. The thymus almost entirely disappears, and the spleen, lymphatic glands and lymph nodules in the intestine show definite atrophy.

These changes were not found to be present to any degree in guinea-pig scurvy. The thymus showed little change. The spleen was so variable in weight that no definite conclusions could be drawn. The lymphatic glands were smaller than on the control animals and frequently showed congestion.

On histological examination the lymph paths were normal. The endothelial cells, however, frequently showed pigment granules. These were probably granules of haemoglobin which had been absorbed from areas where blood had been extravasated.

In a communication which appeared while this paper was passing through the press, Cramer and his co-workers [1921, 2] announce that the atrophy of the lymphoid tissues which they have described can occur when vitamin B alone is withheld from the diet, and that it is associated with a diminution of the lymphocytes of the circulating blood, the polymorpho-nuclear leucocytes being unaffected. The absence of vitamin A alone does not produce any of these changes.

Bedson [1921] has recently described the condition of the blood in scurvy. He finds no constant or striking changes in the total or differential leucocyte count.

It would, therefore, appear that in guinea-pig scurvy (as in vitamin A deficiency) no changes are found in the lymphoid tissues analogous to those which occur when vitamin B is withheld.

Summary.

1. Experiments on guinea-pigs are described to determine whether raw cow's milk and orange-juice replace one another in direct proportion to their respective antiscorbutic values.

2. The question is discussed whether the minimum dose which can afford protection from scurvy is reduced when both the above substances are administered simultaneously.

3. The condition of the lymphoid tissues in scurvy is described.

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LXXXVIII. THE ORIGIN OF MILK FAT, AND ITS RELATION TO THE METABOLISM OF PHOSPHORUS.

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(*Received November 25th, 1921.*)

An outstanding feature of the variation appearing in the yield of milk from individual cows for successive days, as shown by the work of Crowther [1905], Hammond and Hawk [1917], and Gowen [1919], is the want of proportion between the weight of the fat and that of the other constituents of milk. Sheehy [1919] has demonstrated that the fat varies from day to day independently of the solids not fat and water.

To explain this phenomenon it is assumed that

(1) Fat is not derived from the same source as the other constituents of milk, or that

(2) It has the same origin but is secreted in quantity independent of the other components of milk through the peculiarity of mammary gland metabolism.

An experiment to elucidate this matter was conducted at the Albert Agricultural College, Dublin. It was sought to compare the secretion of the mammary gland when supplied with blood rich in absorbed fat with that produced when carbohydrate was assimilated. A good milking cow, No. 71, in moderate condition, receiving the usual daily ration of the herd, *i.e.* roots 80 lbs., concentrates 5 lbs. (fed in three equal portions at 6 a.m., 11 a.m., and 3 p.m.) and hay given *ad lib.*, was milked five times a day—at 6 a.m., 9 a.m., noon, 3 p.m., and 6 p.m., for seven days. She was fasted for the last eighteen hours of the seventh day, and on the following morning—6 a.m.—was given a feed consisting of

Mangels	36 lbs.
Hay	3 „
Oil (linseed)	1 pint

and got nothing except water for the remainder of the day. She was milked as during the previous week, and the weight of milk and the percentage and weight of fat determined as before. The yield at 9 a.m., 12 noon, 3 p.m., and 6 p.m., of the eighth day was taken as representing the manufactured product of the mammary gland when supplied with blood containing a large quantity

Table I

		6 a.m.			9 a.m.			Noon			3 p.m.			6 p.m.			Total daily milk (lbs.)	Total daily fat (lbs.)
Day	Food	Milk (lbs.)	Fat %	Fat (total lbs.)	Milk (lbs.)	Fat %	Fat (total lbs.)	Milk (lbs.)	Fat %	Fat (total lbs.)	Milk (lbs.)	Fat %	Fat (total lbs.)	Milk (lbs.)	Fat %	Fat (total lbs.)		
1	Normal	19¼	3·6	0·693	1	7·6	0·076	3½	3·4	0·119	8	4·6	0·368	3	4·3	0·129	34¾	1·385
2	,,	14	3·2	0·448	¾	6·9	0·052	4¾	3·8	0·181	3½	4·8	0·168	3	4·5	0·135	26	0·984
3	,,	14	3·1	0·434	1¼	4·5	0·056	4½	4·3	0·194	4	4·3	0·172	3½	4·9	0·172	27¼	1·028
4	,,	15¼	3·1	0·473	2	4·7	0·094	3¾	3·9	0·146	4¼	4·5	0·191	2¾	5·4	0·149	28	1·053
5	,,	16½	3·1	0·512	2¾	3·7	0·102	3½	3·9	0·127	4½	5·9	0·266	2½	4·7	0·118	29½	1·125
6	,,	7½*	2·3	0·173	7¾	3·4	0·264	4½	5·7	0·256	3¾	5·6	0·210	2¼	4·5	0·101	25¾	1·004
7	Fast from noon	8*	2·8	0·224	3¾	5·4	0·203	3¼	4·6	0·150	3¾	4·9	0·184	3	3·2	0·096	21¾	0·857
8	Fat	12½	2·8	0·350	4½	4·8	0·204	2¼	5·4	0·122	3	4·6	0·138	½	3·6	0·018	22½	0·832
9	Normal	10	1·9	0·190	7¾	4·5	0·349	2¾	4·9	0·135	3	4·2	0·126	4¼	3·5	0·149	27¾	0·949
10	Fast from noon	10	2·6	0·260	5¾	4·7	0·270	3¾	5·3	0·199	2½	5·4	0·135	3¼	4·4	0·143	25¼	1·007
11	Molasses	15½	2·7	0·412	3	5·1	0·153	3½	5·3	0·172	3	4·8	0·144	2½	4·1	0·103	27	0·984
12	Normal	11	3·2	0·352	3	4·6	0·138	2¾	4·4	0·121	3	5·1	0·153	2½	3·9	0·098	22¼	0·862

* There was no apparent reason for small yields on these two occasions. Such abnormal yields did not appear in the repeated experiments.

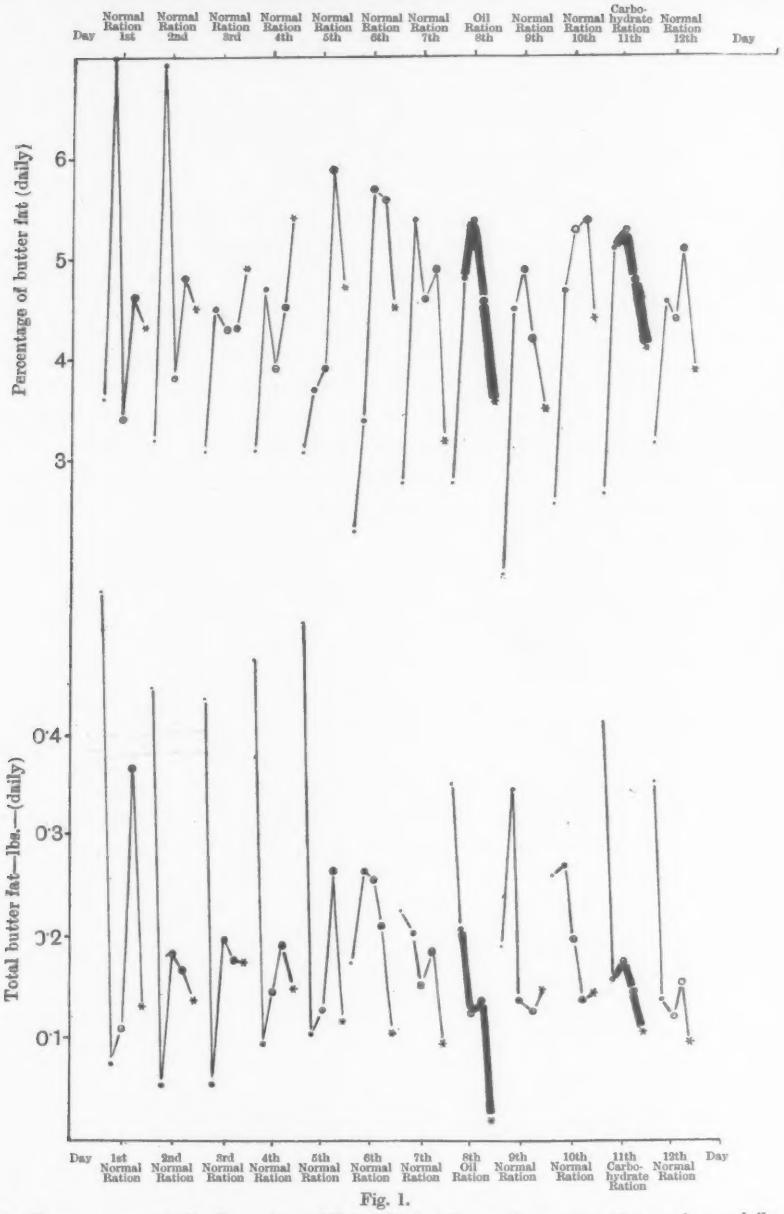

Fig. 1.

Top diagram represents the Percentage of Fat yielded at 6 a.m., 9 a.m., noon, 3 p.m., 6 p.m. daily
Lower „ „ Total weight „ „ „ „ „ „

Index · 6 a.m. ● 9 a.m. ⊙ noon ⊕ 3 p.m. * 6 p.m.

of absorbed fat. The cow got her usual feed on the ninth day and up to and including her noon meal on the tenth day. At 6 a.m. on the eleventh day, after an eighteen hours' fast, she was given the following feed:

Mangels	36 lbs.
Hay	3 „
Molasses	5 „

The milking, five times a day, was continued up to the twelfth day.

The results obtained are tabulated in Table I and plotted in Fig. 1. The graphs on Fig. 1 enable a comparison to be made between the milk-fat produced from blood containing much neutral fat in suspension and that from blood not so enriched. The heavy lines join the milk-fat yields, percentage and total, given at 9 a.m., noon, 3 p.m., and 6 p.m., on the eighth and eleventh days when the possible effects of the fat and carbohydrate fed would be respectively shown. The character of the graphs for these two days is similar, showing that the absorption of fat from the intestine affects the percentage and the total quantity of fat in the milk produced in the same way as the absorption of equidynamical proportions of carbohydrate. If the production of milk-fat were to depend on the proportion of neutral fat floating in the blood the percentage fat in the milk yielded on the eighth day should be higher than that of the eleventh day unless it is assumed that all the carbohydrate absorbed on the eleventh day is directly converted into neutral fat and delivered into the blood as quickly as fat is poured into it from the thoracic duct on the eighth day, an assumption which is in contradiction to our knowledge of general metabolism. But even the adoption of this premise would not explain why, in a repeated experiment, when double the dose of fat was given on the eighth day there was no higher percentage of fat in the milk than on the days when the cow was normally fed or fasted from noon, in which case the fat fed on the eighth day was greater than the fat equivalent of the ration of the other days. Presumably the immediate source of milk-fat is some other chemical compound of the blood, and for the production of this compound fat and sugar can replace one another. Evidence from another experiment conducted by the writer, not yet published, shows that fat and carbohydrate can replace one another for the production of milk-fat over protracted periods.

The experiment was repeated on two other cows, Nos. 68 and 70. On the eighth day at 6 a.m., after an eighteen hours' fast, they were fed as follows:

Cow No. 68.		*Cow No.* 70.	
Mangels	36 lbs.	Mangels	36 lbs.
Hay	3 „	Hay	3 „
Linseed oil ...	1 pint	Molasses	5 „

The yields were recorded as before and on the eleventh day at 6 a.m., after a similar fast, they were again fed the special diets this time as follows:

Cow No. 68.		*Cow No.* 70.	
Mangels	36 lbs.	Mangels	36 lbs.
Hay	3 „	Hay	3 „
Molasses	5 „	Linseed oil ...	1 pint

The relative effects of carbohydrate and fat could thus be compared for one cow on different days and for two cows on the same day. The results from these two cattle led to the same conclusion as those from No. 71. The experiment was repeated with cows Nos. 68 and 70 with margarine instead of oil and this gave a similar result. Lest the amount of fat fed in these instances was insufficient the experiment was again repeated this time on cows 52 and 76; the procedure was similar to that with 68 and 70 except that the quantities of fat and carbohydrate given with the special feeds on the eighth and eleventh days were increased. On the eighth day the cows were fed as follows:

	Cow No. 52.			*Cow No.* 76.	
6 a.m.	Mangels ...	25 lbs.	6 a.m.	Mangels ...	25 lbs.
	Hay	2 „		Hay ...	2 „
	Oil	1 pint		Molasses ...	5 „
9 a.m.	Mangels ...	15 lbs.	9 a.m.	Mangels ...	15 „
	Hay	1 lb.		Hay ...	1 lb.
	Margarine ...	1 „		Starch ...	$2\frac{1}{4}$ lbs.

and on the eleventh day the feeds were reversed, *i.e.* 76 got the ration previously fed to 52 and *vice versa*. The results led to the same conclusion as those from the previous experiments.

In the case of sugar it has been shown that the glucose of the blood [Röhmann, 1919] diffuses into the mammary gland cells where it is converted into the less diffusible lactose of milk. It is highly probable that, in like manner, the non-diffusible fat of milk is made from some diffusible fatty compound. The phosphatides of the blood are soluble compounds containing complex groupings of fatty acids, carbohydrate, phosphorus and nitrogen. In fact, Meigs, Blatherwick and Cary [1919, 1920] have shown that the phosphatide content of the blood of the mammary vein in a lactating cow is less than that of the jugular vein, and conclude that the mammary gland utilises some of the phosphatides of the blood as it passes through it.

Since the phosphatides contain definite proportions of phosphorus, nitrogen and fatty acid groupings in their constitution, their metabolism in the mammary gland should give rise to constant proportions of phosphorus and fat in milk. It must be remembered, however, that the inorganic phosphorus is diffusible whilst fat and some of the nitrogen compounds of milk are not, and it is conceivable that part of the phosphorus might diffuse back into the blood leaving fat and other non-diffusible compounds imprisoned in the gland. Possibly some of the phosphorus liberated is utilised in the synthesis of caseinogen, but in any case if all the inorganic phosphorus derived from the disintegration of phosphatides in the gland cells were secreted in the milk

there should be some definite relationship between the content of either the total or inorganic phosphorus and the content of fat or caseinogen.

To investigate this matter the following percentage determinations were made in milk from individual cows over successive days: fat, caseinogen, total and inorganic phosphoric acid. Table II gives a few of the results:

Table II.

		% fat	% caseinogen (Van Slyke)	% total phosphoric acid	% phosphoric acid in caseinogen (calculated from Richmond's formula for caseinogen)	% inorganic phosphoric acid
Cow 15	May 4	2·7	2·88	0·208	0·108	0·100
	„ 5	3·1	2·90	0·218	0·109	0·109
Cow 135	„ 4*	2·0	2·16	0·214	0·081	0·133
	„ 5*	8·0	2·76	0·215	0·104	0·111
Cow 34	„ 4	3·2	2·59	0·236	0·097	0·139
	„ 5	5·2	2·53	0·225	0·095	0·130
Cow 76	„ 4	2·8	2·93	0·194	0·110	0·084
	„ 5	2·8	2·55	0·162	0·096	0·066
Cow 73	„ 8	3·4	—	—	—	—
	„ 9	3·4	—	—	—	—
	„ 10	3·4	—	0·217	—	—
	„ 11	3·3	—	0·212	—	—
	„ 12	3·4	—	—	—	—
	„ 13	3·5	2·53	0·216	0·095	0·121
	„ 14	3·3	—	0·210	—	—
	„ 15	3·1	2·41	0·214	0·090	0·124
	„ 16	3·5	—	—	—	—
Cow 52	„ 8	3·8	—	—	—	—
	„ 9	3·5	—	—	—	—
	„ 10	3·2	2·83	0·177	0·106	0·071
	„ 11	—	—	—	—	—
	„ 12	3·8	—	0·175	—	—
	„ 13	3·4	—	0·197	—	—
	„ 14	3·5	—	—	—	—
	„ 15	3·8	2·44	0·187	0·092	0·095
	„ 16	3·4	—	—	—	—

* Selected because of marked difference in the percentage of fat on two consecutive days.

There certainly is no constant relationship between the percentage of phosphorus secreted in milk and the quantity of either fat or caseinogen. An increased secretion of fat or caseinogen or both is not always accompanied by an increased amount of phosphorus, the secretion of which appears to be influenced by some other conditions. Since a large increase in the fat secreted is not accompanied by a corresponding increase in the phosphorus, evidently some of the inorganic phosphorus must be reabsorbed from the mammary gland into the blood, and possibly variation in the permeability of the gland cells regulates the secretion of inorganic phosphorus in the milk.

The two experiments quoted lead to the following conclusion regarding the metabolism of the mammary gland: soluble phosphatides diffuse from the circulating blood into the gland cells, are disintegrated there to produce phosphorus, fat, and other products. The non-diffusible products remain in the gland and are afterwards secreted as milk with part of the soluble phosphorus, some of which meanwhile diffuses back into the blood. This view is supported by the work of Meigs, Blatherwick and Cary [1919, 1920] who on comparing the blood of the mammary vein with that of the jugular found that the inorganic phosphorus content of the former was greater than that of the latter. Evidently the mammary gland had elaborated part of the phosphatide of the blood and had returned to the mammary vein inorganic phosphorus.

Summary.

1. For the manufacture of milk-fat, carbohydrate and fat can replace one another in the food. Presumably the immediate source of milk-fat is a diffusible compound to be found in the blood, and there is evidence that the precursor is phosphatide.

2. The phosphatides diffuse from the blood into the mammary gland cells, are there elaborated, and phosphorus, fat and other substances are produced; the non-diffusible products, fat, etc., are all secreted into the lactiferous tubules; the diffusible inorganic phosphorus is partly secreted into the milk and partly diffused back into the blood.

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LXXXIX. STUDIES OF THE COAGULATION OF THE BLOOD.

PART I. SOME PHYSICO-CHEMICAL ASPECTS OF COAGULATION.

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Possibly no better example could be given of nomenclature being in advance of actual knowledge, than the literature of blood coagulation; but despite the multiplicity of alleged substances invoked and considered essential, it will be agreed, it is thought, that a complete explanation is still wanting. The purpose of this communication is to investigate how far the mechanism of blood clotting is susceptible to some of the current concepts of physical chemistry.

For convenience we can group modern theories on coagulation under four headings:

(1) *Wooldridge's theory.* Here coagulation is thought to be a reaction between the constituents of the plasma; the extra-vascular coagulant fibrinferment (thrombin) being a subsidiary reaction product. Wooldridge considered "lecithin" to be of great importance, but he also regarded the plasma as a unity in its behaviour in coagulation, which process he believed to be allied closely to crystallisation.

(2) "*Thrombin*" *theories.* Prothrombin is assumed to exist in, and to be liberated from leucocytes; on activation by calcium, thrombin is formed and is the immediate cause of coagulation [Schmidt and others, 1875]. The term thrombogen is employed by Morawitz [1904] and Fuld and Spiro [1904] in place of prothrombin; this under the combined action of thrombokinase (from tissue detritus) and calcium salts, yields thrombin. Fuld and Spiro regarded coagulation essentially as a proteoclastic digestion. To account for the normal fluidity of blood it was found necessary to postulate the existence of antithrombin [Howell, 1911]. Even then the recognised facts cannnot be explained and the aid has been sought of further hypothetical substances, viz. prothrombokinase, antithrombokinase, antiprothrombin [Collingwood and Macmahon, 1912], proantithrombin [Howell and Holt, 1918], and in serum an inactive compound of thrombin and antithrombin termed by Morawitz [1904] metathrombin. Emphasis should be laid on the fact that thrombin

has not been found in normal circulating blood, but exists only in shed blood. Further, as shown by Wooldridge [1893], on massive injection it fails to induce intravascular coagulation. This latter is a crucial experiment, and unless an instantaneous production and liberation of antithrombin (so far unproved) is admitted, it is thought that doubt is cast on the theories grouped under this heading.

(3) *Theory of Nolf.* Broadly speaking, the views of Nolf [1906, 1908, 1909] may be described as based on the brilliant deductions of Wooldridge. They were fundamentally influenced, however, by his adherence to the "thrombin" theories, particularly in this author's later work. In his earlier papers, Nolf described coagulation as being essentially a mutual precipitation of colloids. He considered that this process involved a proteoclastic enzyme termed leucothrombin derived from the leucocytes and the endothelial cells of the vascular system as well as two proteins formed in the liver, hepatothrombin and fibrinogen. In the presence of calcium salts leucothrombin and hepatothrombin unite to give thrombin which in turn precipitates fibrinogen as fibrin. Excess of hepatothrombin he claimed, leads to the fluidity of normal circulating blood and of "peptone" plasma. He identified hepatothrombin with antithrombin. Nolf also maintained the existence of two forms of coagulation, that produced by thrombin in solution yielding a gel, and that caused by finely divided solid thrombin forming agglutinations. Later, to bring his work into line with that of Morawitz, he termed leucothrombin thrombozyme and hepatothrombin thrombogen. He changed his opinions also regarding the fluidity of both "peptone" plasma and normal circulating blood. The former phenomenon he ascribed to another hypothetical substance claimed to be secreted by the liver, which he called "liver antithrombin." Normal fluidity he described as due to the living cells of the blood and those lining the vascular and lymphatic systems being covered with a film of "fibrin" containing thrombozyme, thrombogen and fibrinogen. Thrombin, he stated, acts only through the medium of thromboplastic substances. The protective film having no thromboplastic action, the blood remains fluid. The preliminary act of coagulation is said to arise from the contact of either thrombogen or thrombozyme with fibrinogen. Enzyme action is claimed subsequently to occur and is termed fibrinolysis; this causing stable clotting. To round off this now somewhat complex theory one last anticoagulant substance termed antifibrinolysin is introduced. It is assumed to be formed by the liver.

(4) *The theory of Hekma.* This hypothesis is based on the close similarity of the precipitate of fibrin alkali hydrosol to fibrinogen, from which Hekma [1916, 1, 2, 3] concluded that fibrin exists in the blood and that coagulation is a colloidal precipitation occurring in two phases, (*a*) a change from emulsoid to suspensoid state with the appearance of ultramicroscopic crystalline-like needles (as observed by Stübel [1914]), and (*b*) the formation of fibrin threads by apposition of these formed elements. One may remark that Hekma does not account for the formation of thrombin in shed blood, the occurrence of

which indicates, with some precision, a chemical as well as a purely physical reaction. Further, he does not take into account the "negative phase" of blood coagulation and other seemingly anomalous phenomena.

The Negative Phase.

The delay in coagulation following slow injection of tissue extracts, first noted by Wooldridge, is one of the strongest arguments for the occurrence of antithrombin. It has been shown, however, by one of us (J. W. P.) that on injection into dogs, cats or rabbits, synthesised colloids free from phosphorus behave similarly to animal extracts. Mills [1921] has recently shown that in blood withdrawn during the "negative phase" there are no substances present which inhibit the coagulation of normal blood; this author and Gutmann [1914] suggest that the "negative phase" is due to the removal of fibrinogen from the plasma, as a diminished content of fibrinogen is found in fluid blood after establishment of the "negative phase." Mills also noted that during the "negative phase" the alkali reserve is substantially diminished. There is therefore, if this is correct, after the injection of small amounts of tissue extracts (*a*) a diminution of fibrinogen, (*b*) a lowering of alkali reserve, (*c*) a loss of power to clot. Bearing in mind the action of CO_2 on peptone plasma (*vide infra*), we suggest that union occurs between tissue extracts or colloids injected, those substances forming the alkali reserve, and finally with fibrinogen. The deductions of Henderson [1921] that blood is a single physico-chemical system tend rather to support this conception.

We have investigated some of the physical conditions governing the precipitation of two typical colloids, gelatin and soluble starch, by alcohol and ammonium sulphate. The amount of precipitate of either colloid depends not only on the volume of alcohol or salt added, but also on the rate at which it is added. When the precipitating agent is added slowly, drop by drop, over a long period of time (2–3 hours), the amount of precipitate formed is much less than if the same volume of the solution is added rapidly. To quote from one series of experiments: To each of two flasks containing 50 cc. of 1 % gelatin, were added 60 cc. alcohol as follows:

A. Drop by drop, time taken 2 hours.
B. As rapidly as the burette would permit.

The precipitates were collected, washed and the nitrogen content determined. The precipitates contained the following amounts:

A. 0·043 g. N.
B. 0·086 g. N.

It may be remarked that the less precipitation following the slow addition of the alcohol may be due to the colloid remaining in a high state of dispersion. Similar results were obtained with the precipitation of soluble starch by ammonium sulphate, a result in which electrical phenomena probably play a part (cf. Mines' observations referred to below). Attention may be drawn

to the fact that temperature plays an important part in this result. At relatively high room temperatures (18–22°) the difference is most marked, at low temperatures (0°) little or no difference can be detected even when the reaction is spread over long periods. Apart from the actual amount of the precipitates formed in the two cases their appearance differs. When the precipitating reagent is added slowly the precipitate is in a very fine state of aggregation, whereas when added rapidly the precipitate formed is much coarser. In this experiment we have established a condition analogous to the production of the "negative phase" under conditions which preclude the formation of an antibody. We have found also that if filtered tissue extract is added slowly, drop by drop, over a period of from 20–30 minutes to unsalted birds' blood *in vitro* a temporary "negative phase" (or retarded positive phase) occurs in its coagulation; while an equal volume of the same tissue extract added rapidly causes rapid coagulation. The clots formed after the appearance of this temporary "negative phase" and resulting from the slow addition of tissue extract are looser and more gelatinous than those formed by its rapid addition. When the concentration of tissue extract was twice as strong we failed to obtain evidence of a negative phase even though the extract was added very slowly in small drops (*vide* protocols). These results are not without parallel in the work of other investigators. Experimenting with inorganic sols Spring [1900], Höber and Gordon [1904], Paine [1911], Galecki [1912] and others have shown that the question "as to whether a given concentration of an electrolyte will produce coagulation or not, depends, within a certain range, on how the electrolyte is added, *e.g.* slowly, drop by drop, or rapidly, with very gentle stirring or violent stirring, etc." [Burton, 1916]. Apparently the precipitation of gelatin (due to the hydrophile action of the alcohol) obeys the same law. Dansyz [1902] noted that when ricin or diphtheria toxin was brought into contact with the corresponding antibody, the degree of neutralisation depended upon the method of preparing the mixture, in the sense that when the toxin was added to the antitoxin in two fractions with a time interval between, the resultant mixture contained a much greater amount of free toxin than if the total toxin had been added at once to the antitoxin. These results were confirmed as to diphtheria toxin by v. Dungern [1904], and were attributed by him to the formation of a supposed substance "*epitoxonoid.*" Similarly, if arsenious acid is added in just sublethal amounts to ferric hydroxide it is found [Hewlett, 1921] that to render the mixture toxic, arsenious acid must be added in amounts greater than the toxic dose of the arsenious acid alone. We submit that these phenomena are of one class and are explicable in the same manner. The precipitation of gelatin or starch and the neutralisation of arsenious acid by ferric hydroxide do not admit of any explanation other than physico-chemical, and it is improbable that any antibody is formed in birds' blood *in vitro* by the slow addition of thromboplastic material. If this view is accepted, the necessity disappears for assuming the existence of antithrombin, antifibrinolysin, antiprothrombin, proanti-

thrombin, prothrombokinase and metathrombin, and thus a purely physico-chemical explanation of the coagulation of the blood must be sought.

So far as we are aware, all previous attempts to produce a "negative phase" *in vitro* have failed. The failures are explicable by the fact that salted plasmas were used and the salts inhibited coagulation; that attention was not paid either to the speed of delivery of the tissue extract, its concentration or to the temperature at which the reaction was carried out. Unfortunately no exact method is available for determining the actual concentration of thromboplastic material in a given solution of tissue extract, so that to obtain the negative phase referred to above, observations must be made with varying concentrations, until the correct strength is obtained.

The effect of rise of Temperature on Birds' Blood in vitro.

In the course of our experiments on birds' blood in clean glass vessels we raised the temperature to 37° thinking from our experience in the formation of a "negative phase" in the precipitation of gelatin that this phenomenon might be modified in comparison with experiments at laboratory temperatures. We found, however, that at 37° birds' blood clotted rapidly without the addition of any tissue extract. Control experiments with blood from the same animal in vessels cleaned in the same manner, but maintained at room temperature, showed the blood to remain fluid for several hours. It is well known that mammalian blood will remain fluid *in vitro* at 0° and it appears that the cooling of birds' blood to room temperatures is an essential factor in its fluidity *in vitro*. The difference in the behaviour of mammalian and birds' blood *in vitro* appears to be one of degree and to be a function of temperature.

The Fluidity of normal circulating Blood.

Much ingenuity has been expended by those who maintain the existence of platelets in circulating blood in explaining why the disintegration of these, and other formed elements, does not cause thrombosis, since in their breakdown thrombokinase is liberated into the blood stream. We suggest that parallels exist with the phenomena seen in the slow injection of tissue extracts into an animal, in the slow addition of alcohol or ammonium sulphate to a precipitable colloid and the slow addition of tissue extract to birds' blood *in vitro*, for it can be assumed justifiably that normally there never is massive disintegration of formed elements in the blood stream. We think, therefore, that this disintegration *in vivo* being equivalent to a slow injection of tissue extracts, the action would be the reverse of coagulant, and to the physical properties of these substances and of those of the plasma we may look for the protection of the plasma from intravascular coagulation. If the liberation of thromboplastic material is rapid its hydrophile properties overcome the initial inhibition produced by the introduction of small amounts of tissue extract and thrombosis occurs. We have again a parallel to the precipitation of gelatin

and starch by alcohol, for it is found that after adding slowly a small quantity of the precipitant, when a larger amount is added subsequently a precipitate is formed immediately.

The Liver and Antithrombin.

The rôle played by the liver in the production of antithrombin has been deduced largely from experiments wherein "peptone" has been employed. Gley and Pachon [1895] with impaired liver, and Hédon and Delezenne [1896] with an Eck's fistula showed that on injection peptone is without action. In this connection one can recall the experiments of Lahousse [1889] in which removal of or damage to the liver diminishes greatly the carbon dioxide content of the blood. The experiments of Wright [1894] and of Mathieu and Urbain [1874] demonstrate that a decrease of carbon dioxide tends to lessen coagulability, while an increase produces an exalted tendency to clot. The classical experiment of Wooldridge on the effect of passing carbon dioxide through peptone plasma can also be quoted, when coagulation at once takes place. From the results of these workers clearly it is evident that carbon dioxide is of utmost importance in the clotting or otherwise of plasma, and we suggest that the action of the liver in promoting the fluidity of the blood depends on the variations in the carbon dioxide content of blood arising under various conditions, normal or otherwise. Further, it would seem a simpler conception to assume a union of peptone with plasma components to take place, rather than the production of an antibody the existence of which is unproven. Regarding work directed specifically to the demonstration of the formation of antithrombin by the liver, the researches of Doyon [1912] may be quoted as typical. From this author's protocols it is evident that the clotting time of perfused blood varies with the state of the liver. When the liver is nearly normal, the coagulation time is shorter, and as the liver becomes abnormal the time is lengthened. Further, Doyon obtained antithrombin by such methods as freezing and thawing the liver, and then heating to 100° prior to extraction, and in the case of one animal (the rabbit) only by autoclaving at 120°. This technique appears to be in the highest degree unphysiological, and as evidence for the secretion of antithrombin by the *normal* liver, may be dismissed. The deduction the present authors make from Doyon's experiments is that the normal liver is less effective than the dying or dead organ in producing an anticoagulant. Attention should be drawn also to the recent work of Menten [1920], where the perfusion apparatus was maintained at 38°, but where the liver itself was at a lower temperature. The salient features of these experiments are:

(*a*) Perfusion of the liver with 0·85 % NaCl yielded no anticoagulant.

(*b*) Continuous perfusion of large amounts of Witte's peptone (9 % in 0·85 % NaCl) failed to produce a substance capable of diminishing the time of coagulation.

(*c*) Perfusion of small amounts of peptone, separately and slowly, through

the liver gave, at first, perfusates producing immediate clotting; subsequent perfusates delayed coagulation and finally the later perfusates caused accelerated rate until coagulation was again immediate.

(*d*) During perfusion with peptone the acidity steadily increased throughout the experiment.

An analogy may once more be drawn between the results recorded above on the precipitation of gelatin by alcohol, and the continuous perfusion of peptone in large quantities, and when perfused in small amounts; these experiments in no way militate against the suggestion made above that the action in the liver is on the peptone rather than the formation of a specific anticoagulant. We are at present carrying out experiments on this point.

Experiments with Blood surrounded by Oil.

It is well known that if blood is withdrawn directly into oil the length of time elapsing before coagulation is increased. In the course of observations on the thromboplastic properties of various substances obtained by extracting fibrin, this method was employed, and in some of the initial experiments what appeared to be a reversible stage in the formation of the clot was manifest. The point was investigated in some detail. Using as the test of reversibility the solubility or otherwise of the coagulating blood in tap-water, it was evident, that prior to the production of the typical clot in both mammalian (cat and rabbit) and frog's blood, there is formed a reversible gel. The duration of this phase is very short in mammalian blood, but persists for a relatively considerable time in that of the frog; the latter animal is best suited for observations on the point in question. The following are examples:

(1) Anaesthetised brown rabbit. A. C. E., paraffined cannula in carotid. Coagulation time of blood on clean glass; commencement of clot 4 minutes, clot complete 5·5 minutes. Blood received into oil in paraffined vessels; formation of gel observed after 2 hours 27 minutes, typical clot in 2 hours 37 minutes. Room temperature 15°.

(2) Anaesthetised cat. A. C. E., pithed, artificial respiration. Coagulation time on clean glass through paraffined cannula 6 minutes. Blood received into oil in paraffined vessels; formation of gel 41 minutes, typical clot in 54 minutes. Room temperature 15°.

(3) Frog pithed. Paraffined cannula in aorta. Coagulation time of blood on clean glass 15 minutes.

April 12th. Blood withdrawn into oil in paraffined vessels at 3 p.m.
,, 13th. Formation of gel noted at 3 p.m.
,, 14th. Microscopic examination at 3 p.m. showed corpuscles mobile on slight pressure of cover glass. No rouleaux.
,, 15th. Typical clot formed with entangled corpuscles and imperfect rouleaux.

When in the intermediate stage, blood transferred to oil in which thromboplastic material was dissolved clotted completely in 10 minutes.

The gel stage is soluble in tap-water and the solution after rapid centrifuging shows, in mammalian blood, usually a few fibrin filaments with the débris of corpuscles; in frog's blood no filaments are visible. Microscopic examination of this reversible stage reveals the corpuscles freely movable on slight pressure on the cover glass. There is neither formation of rouleaux nor appearance of crenation, and in good specimens the leucocytes exhibit amoeboid movement. *Pari-passu* with the formation of fibrin filaments the production of rouleaux occurs. It appears therefore that the coagulation of blood surrounded by oil is a progressive change, in which the formation of a reversible gel precedes that of a true clot. In view of the length of time for which this stage persists in shed blood of the frog it is thought that there is evidence for believing thrombin to be formed after this stage, and that coagulation commences by gelation of the colloidal aggregates of the plasma (a physical reaction), and ends in a chemical reaction in which fibrin and thrombin are formed. In this connection one may recall the fact that blood shed directly into alcohol does not yield thrombin, and that the experiments of Howell [1916] indicate that the coagulation of fibrinogen by thrombin cannot be referred to an electrical reaction between oppositely charged particles. Further, from observations on the cataphoresis of oxalated plasma, Howell has suggested that fibrinogen adsorbs both hydroxyl and hydrogen ions, the adsorption of the former leading to a structureless gel, of the latter to precipitation by aggregation. If we can regard the adsorptive changes in shed blood as similar to those occurring in oxalated plasma, it would appear that the first stage in coagulation is the adsorption of hydroxyl ions.

Comments on some Phenomena of Coagulation.

(1) *The action of a thread introduced into blood.* As is known this produces thrombosis in circulating blood and also starts coagulative changes in shed blood. A wetted thread becomes electro-negative; the presence of the negative charge implies the existence of an equal positive charge in the liquid in contact with it, as the other component of the Helmholtz double layer. Some of the colloidal aggregates of the plasma are electro-negative, Lusk [1912] having shown that coagulation of circulating blood takes place at the pole which accumulates negative charges, while serum is electrically inactive [Robertson, 1918]. Ions of the opposite sign to the substance precipitated act as the precipitating agent, as well as diminishing osmotic pressure and viscosity [Loeb, 1921]. The thread wetted by blood produces electrical conditions similar to those which induce precipitation, and coagulation results.

(2) *The action of some organic intravascular coagulants.* The proteins of the plasma are hydrophile and although the surface tension of hydrophile emulsoids may be lower than that of suspensoids, enormous surface tensions remain. The proteins of the plasma are also very susceptible to the action of neutral salts, the concentration of electrolytes determining their physical

state. Tissue extracts, snake venom, and such synthesised colloids as produce intravascular coagulation [Pickering, 1895, 1896], exhibit like properties in varying degrees. Their coagulant action may be explained as due to the alteration of the distribution of the electrolytes (following their injection) modifying the adsorptive unions of the salts of the plasma with the proteins of the plasma. The hydrophile qualities of those coagulants also probably play a part in the disturbance of the colloidal aggregates of the plasma and may be accompanied by the process termed by Hardy [1899] "denaturation." Tissue extracts as shown by Halliburton and Brodie [1894] and synthesised colloids [Pickering, 1896], lose their coagulant action if "purified" by repeated separation and re-solution or if kept too long in either acid or alkaline solution. The explanation of their inactivity may lie in the satisfaction of their adsorptive demands leading to more stable unions with the electrolytes, or by hydrolysis when "purified" by the acetic acid method. The viscosity of these solutions does not appear to be the coagulant factor, as they retain their opalescence and viscosity in their inactive state. Delezenne [1897] showed that tissue extracts produced coagulation *in vitro* in the unsalted plasma of birds and certain teleostean fishes, which indicates that the incoagulability of salted plasmas [Halliburton and Brodie, 1894], is due to the added electrolytes forming a stable union with the plasma and not to any intrinsic difference between circulating and shed blood. Rettger [1909] found that repeatedly washed tissue extracts failed to induce coagulation in birds' plasma *in vitro* but that the addition of either dust or feathers caused this process to take place. This investigator concluded that the so-called "kinase effect" was purely mechanical. The inactivity of washed tissue extracts observed by Rettger appears to us to be similar to the inactivity of tissue extracts and colloids when they have remained for a considerable period in solution or have been "purified." We have found that if a bird's feather be washed thoroughly and subsequently introduced into birds' blood in clean glass vessels, its coagulant action is much less than when the feather has tissue detritus and dust adhering to it. With a washed feather we have observed birds' blood to remain fluid for $1\frac{1}{2}$ hours *in vitro* at ordinary room temperature, whereas with a contaminated feather coagulation may set in in a very few minutes. A feather provides large surfaces which disturb the surface tensions of the colloids of the plasma, leading to the formation of those larger colloidal aggregates which are the precursors of coagulation. Feathers or other substances which are wetted by blood probably also behave in a similar manner to a cotton fibre introduced into the circulation, so that ions of the opposite sign to the electro-negative aggregates of the plasma aid the change from the emulsoid to the suspensoid state. The fact that a clean feather is not so potent a coagulant as thrombokinase is explicable as due to the hydrophile qualities of the latter substance accelerating the change. The relatively slow action of a clean feather in producing coagulation *in vitro* compared with the more rapid action following introduction into the circulation of material capable of being wetted by blood is explicable by

the fact that laboratory temperatures inhibit to some considerable extent the coagulation of birds' blood.

(3) *Salted plasmas.* In salted plasmas inhibition of coagulation is due to the relatively stable unions of the proteins with the added electrolytes; the withdrawal of water from active participation may be also another inhibitory factor. Directly blood is shed it tends to change towards the condition of coagulation. Both sodium chloride and sodium sulphate plasmas clot on dilution, that is, owing to a reduction in the concentration of electrolytes, a resumption of the coagulative process occurs. If excess of salt is added to these diluted plasmas precipitation takes place. We suggest that the change of state from fluid to precipitate or coagulum is a function of the concentration of the electrolytes and that the difference between the precipitate and coagulum is due to the difference in rapidity of action of the electrolytes modifying the adsorptive unions. It will be recalled that attention was drawn to the differences in aggregation of colloidal precipitates according to the rate of addition of the precipitating reagent. Magnesium sulphate plasma does not clot on dilution, as presumably the adsorptive union of this salt and the plasma is more stable than in sodium chloride or sulphate plasmas. The addition of calcium chloride to dilute sodium sulphate plasma hastens clotting and, as shown by Halliburton and Brodie [1894], this salt evokes coagulation in magnesium sulphate plasma. The injection of calcium chloride into the normal circulation increases the tendency of the blood to clot though it does not produce thrombosis. In each of the three cases the disruptive force of the ions from the kation calcium may be the coagulative factor.

Working with oxalate plasma Vines [1921] has shown that increasing amounts of calcium must be added in proportion to the period of exposure to the oxalate. This work falls into line with the fact that tissue extracts and synthesised colloids become inactive by prolonged contact with dilute neutral salts, and also with the fact that slow addition of alcohol to gelatin (which gives a relatively long exposure to the precipitant) increases, at certain temperatures and concentrations, the resistance of the colloid to precipitating action.

Cramer and Pringle [1913] found that if oxalate plasma is filtered through a Berkefeld filter it fails to clot on the addition of calcium chloride, but if the blood is withdrawn at the height of digestion, oxalated and then similarly treated, it does clot. In these and in other experiments with salted plasmas, so far as we can ascertain, the blood was not withdrawn through a paraffined or similarly treated cannula, and consequently in its passage from the animal to the oxalate coagulative changes had already commenced. The first stage in coagulation appears to be the formation of colloidal aggregates which are larger than those existing in circulating blood. The explanation of the observations of Cramer and Pringle may therefore be found in the fact that the Berkefeld filter removes these larger aggregates; this eliminates the foci from which coagulation starts under the influence of the ions arising from the

calcium chloride. In blood withdrawn at the height of digestion we have a marked increase of carbon dioxide, an accelerator of coagulation, as Wright [1894] has shown that injection of calcium chloride into an animal breathing an atmosphere surcharged with carbon dioxide produces universal intravascular coagulation. Moreover, in blood withdrawn at the height of digestion there occurs "blood-dust" (probably ultramicroscopic fat particles); these also may provide foci which aggregate under the influence of ions arising from the carbon dioxide and added calcium chloride.

(4) *The action of addition of small and of large volumes of water to blood.* The addition of small quantities of water to blood hastens coagulation. Howell [1912] found that while dilution with water increases the coagulability of the blood of the terrapin, dilution with an isotonic solution of sodium chloride does not alter it. Howell concluded that dilution with water weakens the action of the antithrombin. It seems to us to be more probable that dilution with small quantities of water disturbs the surface tensions of the colloidal aggregates and thus starts the change from the emulsoid to the suspensoid state, a conclusion which is borne out by the non-coagulative action of the isotonic salt solution. Dilution with large quantities of water inhibits coagulation. The mass action of excess of water not only retains the colloids of the plasma in an emulsoid form, but, we have found, so far stabilises them that on evaporation at atmospheric temperature in a desiccator to the original or even a smaller volume the property of clotting is destroyed. On evaporation to complete dryness the residuum is not in fibrillar form. The power to clot is apparently a function of the water content of the colloidal aggregates, a fact not to be lost sight of in discussing the action of inhibitors of coagulation.

(5) *Dilution with excess of calcium chloride.* Dilution with a considerable excess of this salt inhibits coagulation. Employing a 2 % solution we have found that evaporation at atmospheric temperature does not produce clotting, so far as can be observed, at any stage in the concentration of the liquid. The calcium ions appear to be unable to overcome the initial hydrolysis of the colloidal aggregates occasioned by the mass action of excess of water.

(6) *The rôle of blood platelets.* These are regarded by many physiologists as playing an important part in coagulation. Their very existence in normal circulating blood, however, has been denied. Buckmaster [1906] has pointed out that they may be produced by fixing fluids, by contact with foreign bodies and by lowering the temperature from 37° to 18° or 20°. They accumulate round a cotton fibre introduced into the circulation, and if blood is received into a glass vessel the platelets are found adherent to the glass, coagulation commencing from the walls of the vessel and spreading from without to the centre. Both the fibre and the glass are electro-negative occasioning free positive ions, and these latter being of opposite sign to at least some of the colloidal constituents of the plasma, disturb its condition. The formation of platelets appears to be analogous to the formation of spherules round a thread immersed in a solution of calcium chloride and placed in a boiled egg-white solution as observed by Hardy [1899].

Cramer and Pringle state that if mammalian blood is received into paraffined vessels, intact platelets are found and account for the fact that no coagulation is produced, by assuming that thrombokinase is not liberated. As was suggested above, even in blood remaining in a fluid condition, changes towards coagulation are already in progress. In mammalian blood the platelets appear to be the first visible sign of change and their aggregation when in contact with glass or a thread may be explained by the disturbing effect of altered surface tensions and by the disruptive activity of positive ions. We have no need to assume the liberation of any thromboplastic substance, as the physical forces at work supply a sufficient explanation. The observations of Mines [1912] on the adherence of the positively charged corpuscles to those negatively charged, when the blood of *Scyllium canicula* is mixed with certain concentrations of cerium chloride, afford an interesting comparison. When platelets are influenced by positive ions arising from the contact of the blood with glass or string, their aggregation may be due to a similar cause. We have observed in the first stages of the coagulation of frogs' blood in oil, that the corpuscles move freely in the surrounding gel on slight pressure, later, rouleaux appear, followed rapidly by complete coagulation. In the light of Mines' work, the formation of rouleaux indicates electrical change and it seems it is to electrical phenomena rather than to the liberation of thromboplastic substances that we must look for the initial development of coagulation. The divergence of opinion as to the existence or absence of platelets in normal circulating blood may possibly be explained by their formation when the formed elements of the blood are disintegrating and their absence when this process is not taking place. We have seen that a colloid may be partly precipitated by the addition of a small quantity of an electrolyte and that this precipitate may dissolve rapidly on diffusion of the electrolyte; the solution, moreover, exhibits some resistance to further precipitation. In a similar manner may not platelets dissolve under normal conditions and thus interact with the salts of the plasma to form a substance with anticoagulative properties?

(7) *The action of peptone and of lung extract.* It has been assumed that peptone or some impurity therein activates the formation of antithrombin by the liver. This organ is not, however, an essential factor in the production of fluidity in shed blood, as Pavloff [1887] found that the circulation of a mixture of defibrinated and living blood through a heart lung preparation yielded a fluid which remained uncoagulated for several days. In the following simple experiment the action of the liver was eliminated:

The aorta of a pithed cat was clamped near the heart so that blood passed only through the pulmonary and coronary circuits. Blood from the carotid prior to occlusion of the aorta withdrawn through a paraffined cannula commenced to clot in 3 minutes and was completely clotted in 5 minutes. Seven minutes after clamping the aorta blood withdrawn in the same manner commenced to clot in $9\frac{3}{4}$ minutes and was completely clotted in $14\frac{1}{4}$ minutes.

Under the conditions of this experiment therefore a short "negative phase" was produced.

Mason [1921] has recently drawn attention to the fact that a number of electro-negative substances accelerate coagulation, citing a considerable list which included carbonic acid, while Mills [1921] has stated that lung extract, a powerful intravascular coagulant, is also electro-negative in neutral solution. The experiments of Wright [1894] on the coagulability of blood during the breathing of various mixtures of carbon dioxide and oxygen, tend to show that the latter diminishes coagulability, while the former in certain concentrations increases it. In the experiment detailed above it seems probable that the great increase of oxygen and oxyhaemoglobin was the cause of the retarded coagulation. Oxygen and oxyhaemoglobin are electro-positive and presumably the free ions in the adjacent field are electro-negative, that is, of the same sign as the coagulable constituents of the plasma. We have again an apparent similarity to the phenomena of precipitation, as the presence of free ions of the same sign as that of the substances in solution tends to stability, while free ions of the opposite sign tend to change from the emulsoid to the suspensoid state.

Summary.

(1) In the slow precipitation of gelatin by alcohol a condition has been found closely analogous to the "negative phase" of blood coagulation and to the "Dansyz reaction."

(2) If dilute filtered tissue extract be added drop by drop to birds' blood *in vitro* a temporary "negative phase" (or "retarded positive phase") in its coagulation is obtained. An equal volume of tissue extract added rapidly to the same volume of blood, under otherwise similar conditions, causes rapid coagulation.

(3) The concentration of tissue extract, the speed of its addition and the temperature of the reaction are factors which determine the occurrence of either a "negative phase" or rapid coagulation.

(4) In view of these *in vitro* reactions it is submitted that the necessity is obviated for assuming the existence of the following substances in discussing blood coagulation: antithrombin, proantithrombin, antiprothrombin, antifibrinolysin, prothrombokinase, and metathrombin.

(5) The non-occurrence of thrombosis in normal circulating plasma owing to disintegration of formed elements in the blood is a similar phenomenon to the "negative phase" following the slow injection of tissue extracts and is probably a physical process.

(6) The fluidity of birds' blood in clean glass vessels is a function of its temperature.

(7) In shed mammalian blood and in frogs' blood, the formation of a reversible gel precedes the formation of the true clot. This observation confirms the view of Wooldridge as to the formation of thrombin in coagulation.

(8) Many of the recognised facts of blood coagulation are explicable as physical phenomena.

(9) Attention is directed to the respective electrical charges of accelerators and inhibitors of blood coagulation, also to the similarity of their electrical condition to those associated respectively with precipitation and with maintenance of solution. It is suggested that coagulation of blood commences as a physical and reversible process and ends in a chemical reaction in which there are at least two products, viz. fibrin and thrombin.

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Protocols of experiments with birds' blood.

In all these experiments the blood was withdrawn through a glass cannula into glass vessels treated successively with hot alkali, hot acid, water, alcohol and ether. The tissue extract was prepared from kidney by the sodium chloride method.

(1) *Pithed fowl.* Blood in contact with tissue commenced to clot in 3 minutes 50 seconds; completely clotted in 6 minutes.

To a certain volume of blood 2·8 cc. tissue extract were added as rapidly as the burette would permit at 4.1 p.m. At 4.10 p.m. clots were observed to form. At 4.20 p.m. clotting complete.

To an equal volume of blood 2·8 cc. of the same tissue extract were added slowly from 4.5 p.m. to 4.25 p.m. At 5 p.m. the first signs of clotting were observed. Only at 5.40 p.m. was clotting complete.

An equal volume of blood without the addition of tissue extract remained fluid till observations ceased. Temperature 17°.

(2) *Pithed fowl.* Blood in contact with tissues commenced to clot in 3 minutes 50 seconds, completely clotted in 6 minutes.

To a certain volume of blood 7·3 cc. of tissue extract were added as rapidly as possible at 5.1 p.m. At 5.8 p.m. slight clots appeared. At 5.23 p.m. the clots firm and contracting.

To an equal volume of blood 7·3 cc. of tissue extract were added slowly from 3.58 p.m. to 4.17 p.m. At 4.49 p.m. first signs of clotting observed. At 5.40 p.m. clotting apparently complete.

To blood from the same animal was added a feather. Clotting appeared to commence in 35 minutes and to be complete in 45 minutes.

Blood from the same animal in clean glass had not clotted when observations were abandoned. Temperature 17°.

Repetition of these observations with tissue extract of double the concentration gave no difference between "slow" and "fast" addition of equal volumes to equal volumes of blood. The time of clotting in each case was $4\frac{1}{2}$ minutes.

(3) *Pithed fowl.* Blood in contact with tissue clotted in 7 minutes.

To a certain volume of blood 3 cc. tissue extract were added as rapidly as possible at 3.5 p.m. Clotting first observed at 3.17 p.m., apparently complete at 3.25 p.m.

To an equal volume of blood 3 cc. tissue extract were added from 2.57 p.m. to 3.27 p.m. At 3.52 p.m. first sign of clotting was observed, at 4.2 p.m. the clot was apparently complete.

Blood from the same animal remained fluid in contact with glass until observations were abandoned.

Other experiments of precisely the same nature as above have given the same results in each case.

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XC. THE DISTRIBUTION OF CARNOSINE IN THE ANIMAL KINGDOM[1].

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CARNOSINE, which was first isolated by Gulewitsch [1900], has now been proved to be β-alanylhistidine in two independent investigations [Baumann and Ingvaldsen, 1918; and Barger and Tutin, 1918].

This dipeptide is to be found in all mammalian skeletal muscle and also to a less extent in mammalian cardiac muscle. E. Mellanby [1908] found that creatine is present in vertebrate muscle whilst absent from that of invertebrates, and he suggested that the distribution of carnosine might follow along similar lines. Accordingly a research was made into the distribution of the base in the muscles of various animals. It has been possible to extend the enquiry amongst some of the rarer species, owing to the kindness of Dr Sonntag of the Zoological Gardens.

METHODS USED IN THE RESEARCH.

1. *Colorimetric method.* The quantitative estimation of carnosine in the muscles of the animals investigated was carried out according to the method described by Clifford [1921].

Essentially this consists in making a watery extract of muscle, removing soluble proteins with metaphosphoric acid, and diazotising the resultant protein-free solution. The depth of colour is then matched against a standard in a Duboscq colorimeter.

This method has advantages over the older precipitation methods in that it is rapid and obviates loss of the base by repeated precipitations. Consequently results far higher than those previously given for the carnosine content of muscles have been obtained. This is to be expected since, at every stage of preparation of the pure base, either according to the method of Dietrich [1914] or Gulewitsch [1900], the diazo-reaction shows incomplete precipitation of the base by the reagents used in the final stage. Also part of it is left behind in every one of the huge and numerous precipitates formed in the removal of proteins and inorganic salts.

Therefore, for the estimation of carnosine in a given muscle, the colorimetric method appears preferable to the isolation method. A further advantage

[1] Part of this paper was incorporated in a Thesis for the M.Sc., London.

lies in the fact that small amounts of tissue are sufficient for an estimation. For quantitative isolation of the base, it is useless to work with less than 1000 g. of muscle, whilst colorimetrically 1 g. or even less has been used with success.

2. *Precipitation methods.* Whenever necessary to confirm or supplement the colorimetric readings, a precipitation method has been used.

The necessity for such a procedure was realised on diazotising salmon extract. Since this fish belongs to a carnosine containing family, the Physostomi, it was expected that a positive reaction would be given by the protein-free filtrate.

However, the extract from 12·7 g. of salmon muscle when diazotised gave only a fleeting pink tinge and rapidly turned yellow. On increasing the amount of the extract a red-orange colour resulted which again in a few seconds changed to a deep yellow. When a stronger solution of diazo reagent was added to this yellow liquid fleeting red streaks appeared, which faded almost as soon as they were formed. The presence of carnosine was thus indicated in the solution, but at the same time accompanied by some other substance masking the diazo reaction.

Accordingly, an endeavour was made to obtain pure carnosine from 4 lbs. muscle treated according to the method of Dietrich [1914]. No red colour was obtained on diazotisation until after removal of excess of lead by means of sulphuric acid, when the carnosine reaction became strongly positive in the filtrate. The final re-crystallisation gave a small amount of 95 % pure carnosine.

Pike, salmon-trout, and whitebait gave a similar result to that given by salmon and, therefore, in these four fishes it is impossible to state the amount of carnosine in the muscles, though the base is present, as seen on diazotisation of the lead-free filtrate.

This suggested that other negative results were also due to the presence of an inhibitory substance and therefore 2–4 lbs. of every apparently carnosine-free fish muscle and as much as possible of all other negative muscles was treated according to the precipitation method. The colorimetric findings with the Anacanthini were thus confirmed. Cod, haddock, hake, whiting, brill, halibut, plaice, sole and turbot gave as an end product a sticky mass which on diazotisation gave an intense yellow colour with no trace of red. A similar lack of the base was found with extracts from 100 g. each of oyster and mussel, 30 g. of house-fly and 60 g. of common sparrow.

The actual precipitation method used was as follows.

The tissue was extracted with distilled water at 80–90° for three periods of 1½ hours each. The three extracts were united and the tissue pulp squeezed dry through muslin. A slight excess of lead acetate was added to the hot solution, and the resultant precipitate filtered off. The filtrate was neutralised with 20 % caustic soda at about 45° when another precipitate formed. This was removed, and the filtrate heated to about 60°. It was then freed from excess lead by the addition of sulphuric acid. (At this stage, if carnosine were

present, the diazo reaction of the filtrate became positive even if negative in the original muscle extract.) After filtration the acid solution was neutralised with baryta, re-filtered, and the filtrate evaporated to 50–100 cc. over a water-bath. Absolute alcohol was added till a faint permanent cloudiness appeared. Approximately twice as much alcohol as filtrate was used. A small amount of ethyl ether was added followed by 10 % mercuric sulphate in 5 % sulphuric acid as long as a mercury precipitate formed. After 24 hours the solution was filtered and the precipitate well washed with 5 % sulphuric acid. It was then suspended in water and decomposed by hydrogen sulphide. The mercuric sulphide was filtered off, and the solution neutralised with baryta, excess of this being removed by carbon dioxide. The new filtrate was concentrated on a water-bath to a thick syrup which became crystalline on cooling. This was ground in a mortar with a mixture of four parts of ethyl alcohol and one part of water. The filtrate from this process was evaporated and gave a yield of impure carnosine. The grinding with alcohol and evaporation were repeated twice and in this manner a small amount of 97 % pure carnosine was obtained.

Possibility of Putrefactive Change.

A further suggestion to account for the absence of carnosine from the white fishes was that post-mortem changes had set in and had led to the disappearance of the base. In order to test this, ten sticklebacks were obtained and killed. Five were ground up at once (after removal of the head and intestines) and were extracted with water. The resultant solution when diazotised and examined colorimetrically gave 0·14 % of carnosine for stickleback muscle and bone.

The other five fishes were left for four days exposed to the air at room temperature in the summer. They were then extracted and treated in the same way as the freshly killed ones. Again the yield of carnosine was 0·14 %, showing that keeping fish for four days after death caused no alteration of carnosine content.

One sample of plaice analysed and found carnosine-free had not been dead more than 24 hours when extracted. This estimation was carried out in the winter and extensive bacterial action would hardly have set in in such a short time.

The crayfish, sea anemones, oysters, mussels, scallops, snails, flies and fly maggots were killed immediately before extraction and, therefore, putrefactive changes can be excluded as the cause of the negative diazo reaction in these experiments. With rabbit muscle from (1) a freshly killed animal; (2) a sample sent by post from Cambridge; and (3) a rabbit bought from a poulterer's shop, the yields of carnosine varied from 0·13–0·15 % only. Therefore, it may be said that in fresh muscle with no definite signs of putrefaction the carnosine content remains unchanged at any rate up to four days after death. On the other hand meat kept till definitely putrid gave less carnosine when extracted than the fresh sample.

From these results it was decided to use any sample of muscle from the Zoological Gardens as representing the normal animal unless a smell of putrefaction could be detected. The animals had never been dead more than two days before extraction, but if definite putrefaction had set in the sample was rejected as useless, and no carnosine estimations were made on it.

Constancy of Carnosine Content in the Species.

The amount of carnosine in the muscles of any animal species is constant in every case where it has been possible to make several different extractions. In this it resembles creatine [Myers and Fine 1913]. Owing to this fact, it has seemed legitimate to take a single estimation in the case of rare animals as truly representing the carnosine content of that species.

Actual experiments performed to test this carnosine constancy are as follows.

(1) *Rabbit.*

3	rabbits,	2 days old,	0·135, 0·14, 0·14 %
3	„	4 „	0·13, 0·14, 0·15 %
2	„	28 „	0·15, 0·15 %
2	„	49 „	0·14, 0·16 %
4	„	adults	0·15, 0·15, 0·14, 0·15 %

(2) *Rats.*

2	rats,	30 minutes old,	0·10 %
4	„	2 weeks old	0·11, 0·12, 0·10, 0·10 %
3	„	3 „	0·11, 0·12, 0·11 %
1	„	8 „	0·12 %
22	„	adults	0·10, 0·125 %

(3) *Beef.*

English beef, 6 different samples: 1·1, 1·0, 1·1, 0·98, 1·0, 1·0 %
English veal, 2 different samples: 1·12, 1·05 %

(4) *Sheep.*

English mutton, 3 samples: 0·38, 0·37, 0·38 %
English lamb, 2 samples: 0·40, 0·42 %

(5) *Fish.*

In every case of non-carnosine-containing white fish at least two separate samples were taken and each proved negative on applying the diazo reaction. With cod seven different samples have been tested by two separate methods, always with a negative finding.

Carnosine in Skeletal and Cardiac Muscle.

When possible the carnosine content of cardiac, as well as of skeletal muscle, was investigated. The unexpected result obtained was that in every case the cardiac content was very much lower than the skeletal.

Actual findings were:

	Carnosine in skeletal muscle %	Carnosine in cardiac muscle %
Cockatoo	0·07	0·009
Rhesus monkey	0·34	0·03
Rat	0·11	0·02
Rattlesnake	0·045	Faint trace
Testaceous snake	0·06	„
Sheep	0·38	0·017

This is in contradiction to the findings of Bubanović [1918] who stated that human cardiac and skeletal muscle gave the same carnosine content. However, although he carried out the estimations on human cardiac muscle himself, those on skeletal muscle with which he compares them are those of v. Fürth and Hryntschak [1914] on horse flesh, and the carnosine content of muscle varies enormously in different species.

Carnosine in Red and White Muscle.

With the rat, rabbit and domestic fowl, separate estimations were carried out on samples of red and white skeletal muscle. In no case was any difference of carnosine content obtained.

Distribution of Carnosine in the Animal Kingdom.

(1) *Invertebrates.*

All invertebrate muscle, so far examined, has been free from carnosine. The distribution thus resembles that of creatine which has been shown by various observers to be absent from invertebrates.

A crayfish was killed and 10 g. of its muscle extracted with water, and diazotised after precipitation with metaphosphoric acid as previously described [Clifford, 1921]. No pink coloration resulted, but a yellow was produced. Since a minute trace of carnosine (0·00001 g.) gives rise to a definite pink colour when treated with diazobenzenesulphanilic acid and sodium carbonate, the negative result showed absence of the base from crayfish muscle. This experiment was repeated on two other occasions with similar results.

The following invertebrates have been examined:

	Carnosine in muscle
(1) *Actinozoa.*	
Sea anemone (*Taelia*, 2 varieties)	Absent
(2) *Arthropoda.*	
i. Crustacea.	
Crayfish (*Astacus fluviatilis*)	Absent (2 methods)
ii. Insecta.	
Honey bee (*Apis mellifica*)	Absent
Blue bottle fly (*Calliphora*)	,, (2 methods)
Blue bottle larvae	,, ,,
iii. Arachnida.	
House spider (*Tegenaria domestica*)	Absent
(3) *Mollusca.*	
i. Gastropoda.	
Land snail (*Helix*)	Absent
Sea ,, ,,	,,
ii. Pelecypoda.	
Oyster (*Ostrea*)	Absent (2 methods)
Mussel (*Mytilus edulis*)	,, ,,
Scallop (*Pecten*)	,, ,,

It may, therefore, be concluded that carnosine is absent from the muscles of invertebrates, since neither by direct diazotisation of muscle extracts, nor by an isolation method could a trace of the base be found in any invertebrate examined[1].

(2) *Vertebrates.*

A. Teleostei.

(i) *Non-fatty fishes. Anacanthini.*

Cod muscle was extracted as typifying the teleostean fishes. There was every expectation of a good colour reaction since Suzuki and his co-workers [1909] claimed to have isolated 0·2 % of the base from the bonito, a Japanese fish belonging to the Acanthopteri. However, when the cod extract was diazotised there was no trace of pink in the solution but merely a clear pure yellow. The Japanese observers in their paper state that their "carnosine" prepared from the bonito by precipitation with silver nitrate and baryta gave a negative Pauly reaction. Since pure carnosine when diazotised gives an intense red colour, their final product must have consisted of some other substance.

This negative finding with cod led to the investigation of other Anacanthini with the following results:

Anacanthini	Carnosine in muscle
Cod (*Gadus morrhua*)	Absent (2 methods)
Brill (*Psetta laevis*)	,, ,,
Haddock (*Gadus aeglefinus*)	,, ,,
Hake (*Merluccius vulgaris*)	,, ,,
Halibut (*Hippoglossus vulgaris*)	,, ,,
Plaice (*Pleuronectes platessa*)	,, ,,
Sole (*Solea vulgaris*)	,, ,,

(ii) *Fatty fishes. Acanthopteri and Physostomi.*

The results previously described point to the absence of carnosine from fishes, but when the dark muscled members of two other sub-orders of the Teleostei, viz. the Acanthopteri and Physostomi were examined, they were found to contain considerable amounts of carnosine in their muscles.

With *Acanthopteri* the results were:

Mackerel (*Scomber scomber*)	0·50 % carnosine
Mullet (grey) (*Mugil capito*)	0·18 ,,
,, (red) (*Mullus barbatus*)	0·16 ,,
Sea bream (*Abramis brama*)	0·14 ,,
Stickleback (muscle and bone) (*Gastrosteus pungitius*)	0·14 ,,

[1] This suggested a possible means of identifying the source of commercial fish and meat pastes. On testing it was found that there was a difference between fish and meat paste, for "crab," "anchovy" and "bloater" pastes gave negative reactions, whilst "wild duck" and "ham and tongue" gave 0·50 %. Hence, contrary to general opinion there probably is a true distinction between the two classes of paste, though since "crab" and "bloater" give similar negative results and "wild duck" and "ham and tongue" both gave 0·5 %, there are probably but two basal pastes and no more, for bloaters would be expected to give a positive reaction; and wild duck less than ham and tongue.

With *Physostomi*:

Barbel (*Barbus vulgaris*)	0·18 % carnosine
Eel (*Anguilla vulgaris*)	0·37 „
Gold fish (*Carassius auratus*)	0·11 „
Grayling (*Thymallus vulgaris*)	0·11 „
Herring (*Clupea harengus*)	0·16 „
Minnow (muscle and bone) (*Leuciscus*)	0·09 „
Pike (*Esox lucius*)	Present (precipitation method)
Roach (*Leuciscus rutilus*)	0·18 % carnosine
Salmon (*Salmo salar*)	Present (precipitation method)
Salmon trout (*Salmo trutto*)	„ „
Smelt (*Osmerus eperlanus*)	0·18 % carnosine
Sprat (*Clupea sprattus*)	0·47 „
River trout (*Salmo fario*)	0·11 „
Tench (*Tinca vulgaris*)	0·11 „

Hence, it seems that the distribution of carnosine in teleostean muscle follows the morphological classification, being absent from the Anacanthini and present in the Acanthopteri and Physostomi. It was also present in the one member examined of the

(iii) *Chondrostei*:

Sturgeon (*Acipenser sturio*)	0·23 % carnosine

The classification of these fishes is that of the older zoologists led by Gunther, who put the flat fishes with the other white fishes, *e.g.* the cod and haddock, but in the modern classification they are placed with the Acanthopteri for morphological reasons, the whole system being based on the anatomy of the skull. On chemical grounds, however, the older classification is supported since the flat fishes (Pleuronectidae) lack carnosine in their muscles like the other Anacanthini.

B. Reptiles and Amphibians.

Muscle from several of these animals gave the following results:

(i) *Amphibia.*	
Axolotl (*Amblystoma tigrinum*)	0·19 % carnosine
Frog (*Rana temporaria*)	0·25 „
Frog, young tadpole stage	Present (too little for quantitative estimation)
Newt (*Triton vulgaris*)	0·19 % carnosine
(ii) *Reptilia.*	
Lacertilia.	
Green lizard (*Lacerta viridis*)	0·10 % carnosine
Ophidia.	
American black snake (*Zamenis constrictor*)	Present (too little for quantitative estimation)
Cape Bucephalus (*Dipsas bucephala*) ...	0·03 % carnosine
Corais snake (*Coluber corais*)	0·32 „
English grass snake (*Tropidonotus natrix*) ...	0·20 „
Indian python (*Python molurus*)	0·13 „
Rattlesnake (*Crotalus horridus*)	0·045 „
Testaceous snake (*Zamenis flagelliformis*) ...	0·06 „
Chelonia.	
Speckled terrapin (*Malacoclemmys terrapin*)	Carnosine absent

From these results it seems that carnosine is present in the reptiles and amphibians, but the reptiles, like the fishes, may contain a carnosine-free family. Only one member of the Chelonia, the speckled terrapin, has been examined, and it is carnosine free. However, there was insufficient muscle for the precipitation method to be carried out, and therefore it may be that a substance masking the diazo-reaction, similar to that found in the salmon, was present and prevented the development of the red colour of diazotised carnosine.

C. Birds.

Extracts of bird muscle gave the following results:

(*a*) *Spheniści.*	
Black-footed penguin (*Spheniscus demereus*)	0·055 % carnosine
(*b*) *Falconidae.*	
Sparrow hawk (*Accipiter nisus*)	0·18 „
(*c*) *Columbae.*	
Common pigeon (*Columba livia*)	Trace of carnosine
Southern triangular spotted pigeon	0·033 % carnosine
(*d*) *Paradiseidae.*	
King Bird of Paradise (*Cicinnurus regius*)	0·059 „
(*e*) *Corvidae*	
Blue jay (*Garralus cyanocitta*)	0·043 „
Common jay (*Garralus glandarius*)	0·04 „
(*f*) *Phasianidae.*	
Domestic fowl (*Gallus domesticus*)	0·17 „
Hybrid pheasant fowl	0·02 „
Golden pheasant (*Chrysolophus pictus*)	0·04 „
Partridge, English (*Perdix*)	0·11 „
„ French (*Caccabis rufa*)	0·11 „
Quail (*Coturnix communis*)	0·08 „
(*g*) *Psittacidae.*	
Blue-fronted Amazon (*Chrysotis aestiva*)	0·03 „
Budgerigar (*Melopsittacus undulatus*)	Absent by diazo-reaction, insufficient for precipitation method
Greater sulphur crested cockatoo (*Cacatua galerita*) ...	0·07 % carnosine
Lesser sulphur crested cockatoo (*Cacatua sulphurea*)	0·10 „
Illiger's macaw (*Ara maracana*)	0·04 „
(*h*) *Fringillidae.*	
Bicheno's finch (*Stictoptera bichenovia*)	Carnosine absent
Indigo bunting (*Passerina cyanoa*)	„
Long-tailed finch (*Poëphila acuticauda*)	„
Siskin (*Chrysomitris spinus*)	„
Red-crested cardinal (*Carduelis*)	„
Sparrow (*Passer domesticus*)	„
Tanager (*Rhamphocelus brasilius*)	(Two methods) Carnosine absent
(*i*) *Strigidae.*	
Eagle owl (*Bubo ignavus*)	Carnosine absent

The birds here, as the fishes, fall into two classes, one carnosine-free and the other with carnosine in the muscles. The finches and possibly the owls

resemble the Anacanthini in being carnosine-free, whilst the penguins, birds of prey, pigeons, birds of paradise, jays, game birds and parrots are like the Physostomi, Acanthopteri and Chondrostei in having the base in their muscles.

D. Mammals.

The mammals examined differ from the other big classes in the animal kingdom in that no member of the group has yet been found to be carnosine-free. Estimations were carried out on the following of the mammalia:

(1) *Rodentia.*	
Capybara (*Hydrochaerus*)	0·20 % carnosine
Common marmot (*Arctomys*)	0·06 „
Crested porcupine (*Hystrix cristata*)	0·05 „
Jerboa (*Dipus*)	Present (not enough for quantitative estimation)
Mouse (*Mus domestica*)	0·09 % carnosine
Oak doormouse (*Muscardinus*)	0·09 „
14 specimens rabbit (*Lepus cuniculus*)	0·15 „
32 specimens rat (*Muridae*)	0·11 „
(2) *Ungulata.*	
Antelope (*Anoa*)	0·16 „
6 specimens bull (*Bos*)	1·11 „
2 „ „ (calf)	1·12 „
Horse (*Equus caballus*)	0·90 „
Kashmir deer (*Cervidae*)	0·05 „
Pig (*Sus*)	0·64 „
3 specimens sheep (*Ovis*)	0·38 „
2 „ lamb	0·41 „
(3) *Carnivora.*	
Common fox (*Canis vulpes*)	0·09 „
Leopard (*Felis pardus*)	0·12 „
(4) *Primates.*	
Rhesus monkey (*Macacus rhesus*)	0·34 „

Discussion of Results.

It is difficult to believe that carnosine has little or no significance though it cannot be an absolute necessity since large families of animals exist without it. It is a dipeptide and until recently was the only one known in the body. Another, glutathione, consisting of cystein and glutamic acid was recently isolated by Hopkins [1921] and shown to be a factor in cell respiration.

There are several indications of the importance of carnosine to the organism. It is constant in amount in any given species and is often found in a high percentage. Thus in beef there is 1 % carnosine in the fresh muscle, and since beef is about 66 % water, carnosine is present in the solids to the extent of 3 % whilst even in the pheasant with the low carnosine content of 0·04 % in fresh muscle, the percentage of carnosine in the solids is as high as 0·17 %. It is difficult to believe that a muscle substance of this high concentration should be useless to the organism.

Again carnosine is not found in the urine and therefore the ingested substance must be metabolised as also must that of the muscles. Carnosine is said to be stable to pepsin and trypsin but to be split up by the action of erepsin. This splitting up and subsequent conversion of the β-alanine and histidine to their end products will liberate a definite amount of energy which can be utilised by the organism. In this case, contrary to general opinion, meat extracts which contain from 7–11 % of carnosine are not without any food value to the organism.

Carnosine is the only known substance containing a β-amino-acid in the body and this may be of great importance, but so far the significance of this fact is unknown.

It was hoped that an investigation into the distribution of the base in the animal kingdom would give a clue as to its physiological function, but this has not been the case.

It seemed possible that carnosine might be correlated with muscular activity, but the active cod and the sluggish plaice are alike in lacking it, and the mackerel with similar activities and environment to the cod has a plentiful supply. With birds also the active finches are carnosine-free and the equally active game birds possess the base.

There is again no difference in the carnosine content of red and white muscle though their rate of contraction differs greatly and the heart contains very little of the extractive in comparison with either red or white skeletal muscle. This suggests that carnosine has no functions concerned with oxidations. If it were of importance in this connection it would be expected that the heart, as the most constantly active organ of the body, would contain at least as much, and probably more, than the rest of the musculature.

No connection between diet and carnosine has been found, though it seemed probable that there should be a definite relationship. The carnivorous leopard and the herbivorous antelope have almost the same percentage of carnosine in their muscles, whilst the bull and the sheep, with their practically identical dietaries, show a great difference, the bull containing almost three times more in its muscles than the sheep.

The only relation brought out by this investigation is a morphological one. If the base is absent from one member of a zoological family, it appears to be absent from all. This is well seen in birds where no examined finch has been found to have carnosine.

A similar thing is seen in the fishes under the old classification (with the flat fish in the Anacanthini) where no member of the Anacanthini is found with carnosine, though all the others have a definite amount in their muscles.

It is possible that other extractives such as creatine may also show a selective distribution in the animal kingdom, and if so, generalisations as to the presence of a substance in all muscles, based on estimations of one kind of animal, are unsound.

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XCI. A NOTE ON THE PHYSIOLOGY OF THE SHIP-WORM (*TEREDO NORVEGICA*).

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Chemotaxis.

The duration of the free-swimming larval stage of the wood-boring mollusc, *Teredo*, is a point of some importance in connection with the problem of the protection of timber against its attacks, since it is only during this free-swimming period that infection can be conveyed from one piece of timber to another.

In the course of some experiments with a view to the determination of the maximum length of the larval life of the animal, the larvae were kept in large dishes of sea water in which were placed pieces of wood; it was hoped that some of the larvae might settle on the wood and commence to bore into it.

Although the object was not attained it was noticed constantly during these experiments that there was an aggregation of the larvae upon the surface of the wood. This aggregation took place even when the wood was in the less strongly illuminated part of the dish, although the larvae are normally positively phototropic.

This observation of the apparent tendency of the larvae to aggregate upon the surface of wood, suggested the possibility that a definite chemical attraction for the larvae might be exercised by some normal constituent of wood. The following experiments were undertaken in the attempt to elucidate this point. In the first place some ordinary deal sawdust was extracted in a Soxhlet apparatus with ether for four hours and then with alcohol for a further four hours. Both the solutions on evaporation left a gummy residue, which was thoroughly freed from all traces of solvent. Small patches of these residues were then introduced into dishes of sea water containing some of the larvae, the latter being evenly distributed throughout the dish. After 24 hours the vast majority of the larvae in both cases were closely aggregated around the wood-extracts (see photograph).

In order to determine whether this were only a mechanical effect, the experiments were repeated, substituting in one case small stones, and in another glass beads, for the wood-extracts. In neither of these cases was any attraction to be observed. The further possibility of the extracts exerting a paralytic effect upon the larvae and hence acting as a trap was discounted

by the fact that movement could still be observed even among those larvae which were closely packed around the substance in question.

The results of the experiments were so striking that it was decided to extend them to a number of other substances—both those which might be expected to be toxic and those which might conceivably be present in the wood. Where possible the more dependable method used by Pfeffer for fern spermatozoids was used, which consists in placing a capillary tube filled with a solution of the substance under investigation in a dish with some of the larvae and observing the effect upon the distribution of the latter. The results of experiments with various solvents are shown in the accompanying table and Fig. 1.

Experiments on Attraction of Larvae—Direct Method.

	Substance employed	Effect upon distribution of larvae	Condition of larvae
A	Small pieces of stone	None	Living
B	Glass beads	None	Living
C	Alcoholic sawdust extract	Very marked aggregation of larvae in the course of 24 hours	Living
D	Ethereal extract of sawdust	Same as C	Living
E	Crystal of dinitro-naphthalene	Pronounced aggregation around crystal in 24 hours	Living except immediately around crystal
F	Crystal of chloro-dinitrobenzene	No aggregation after 24 hours	Dead
G	Crystal of T.N.T.	Very slight aggregation	Dead
H	Drop of creosote	Some aggregation	Dead
K	Crystal of calcium malate	Apparent aggregation in both cases, but effect difficult to observe on account of rapidity of diffusion	Living
L	Crystal of calcium succinate		Living

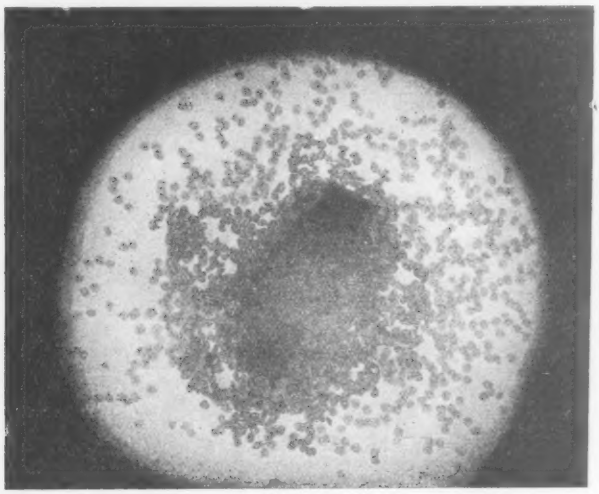

Fig. 1. Photograph showing distribution of larvae in dish about patch of alcoholic extract of wood (×80). After 24 hours.

Experiments on Attraction of Larvae—Capillary Tube Method.

	Contents of tube	Resulting distribution of larvae	Condition of larvae
I.	Sea water	No larvae in tube after 48 hours	Living
II.	Distilled water	A few larvae in tube after 48 hours	Living, except those in tube
III.	Sea water saturated with alcoholic sawdust extract	After 48 hours numerous larvae have entered the tube	Living
IV.	Sea water saturated with ethereal sawdust extract	After 48 hours the mouth of the tube is blocked with a mass of the larvae	Living
V.	Sea water which has been boiled with sawdust	Similar result to IV	Living
VI.	Sea water containing 0·1 % sodium acetate	Very few larvae in tube after 48 hours; after 72 hours some have entered	Living
VII.	Sea water containing 0·1 % sodium citrate	Similar result to VI	Living
VIII.	Sea water containing 0·1 % acetic acid	Large number of larvae enter tube	Living
IX.	Sea water containing 0·1 % hydrochloric acid	Similar result to VIII	Living
X.	Sea water containing 1 % malic acid	There is a rapid re-arrangement of the larvae on introducing the tube into the dish; in a few hours many larvae have entered the tube and many more are gathered about its mouth	Living except for those which have actually entered the tube in XI
XI.	1 % malic acid in distilled water		
XII.	0·1 % malic acid in sea water	Similar to XI and X, but to a less marked degree	Living
XIII.	1 % tartaric acid in distilled water	No perceptible aggregation of larvae	Living
XIV.	1 % citric acid in distilled water	Very slight attraction	Living
XV.	1 % succinic acid in distilled water	No attraction	Living
XVI.	0·1 % vanillin in sea water	No attraction	Living
XVII.	1 % glucose in distilled water	No attraction	Living
XVIII.	Saturated picric acid in distilled water	No attraction	Dead

From these it will be seen that a number of the substances experimented with show a certain power of attraction for the larvae, but that by far the most rapid and intense effect was obtained with malic acid; this acid is one which is very widely distributed in the plant kingdom, and may conceivably be present in the wood; but, without more evidence, it is impossible to express an opinion as to whether this is the substance which is responsible for the apparent attraction exercised by the wood. It is a noteworthy fact that both the alcoholic and ethereal extracts of wood are distinctly acid in reaction, the latter being the more markedly so. The toxic substances used behaved as would be expected, with the exception of dinitronaphthalene; the aggregation which took place around this substance is difficult to explain; probably the substance, which is very slightly soluble in water, acts as a trap for the larvae, those which happen to come within the small area of water in which the concentration of the dinitronaphthalene is toxic, being at once paralysed, and, by making a close ring round the crystal, causing the diffusion of the poison to be still slower, so that the larvae in the other parts of the dish remain unaffected. The aggregation around the drop of creosote may be partly accounted for

in the same way, but in this case vigorous diffusion currents are set up on introducing the oil into the dish, which may also help to account for the phenomenon.

The general conclusions to be drawn from these experiments seem to be as follows:

There is some substance present in wood, and extracted therefrom by water, alcohol and ether, to which *Teredo* larvae are positively chemo-tropic; it is just possible that this substance may be malic acid, but further work is required in order to establish its identity.

Since this positive chemo-tropism exists, the question arises as to what part it plays in the life of the animal.

In view of the natural conditions which prevail in the sea it is not possible to imagine that the larvae can be attracted towards wood by a chemical mechanism, since they must be at the mercy of the wash of the water. It would, however, obviously be of advantage to them if they possessed some means of recognising wood when they came into contact with it.

It would appear from the experiments described above that such recognition is rendered possible by chemical means, and that the larvae are dependent upon this chemical stimulus to enable them to distinguish between wood and stone or other surfaces into contact with which they may chance to be brought. In connection with this phenomenon it is interesting to compare the work of Dakin [1910] on the larvae of Pecten.

The Enzymes of *Teredo*.

The question of the source from which *Teredo* derives its nourishment is an interesting one. The fact that all the wood borings pass through the alimentary canal would naturally lead one to expect that some nutriment would be derived therefrom. On the other hand it has been held that the sole source of food is the plankton which is drawn in through the inhalant siphon and that the wood is passed out of the body chemically unchanged. In order to try and elucidate this point it was decided to make some experiments with a view to the detection, in the digestive gland of *Teredo*, of any ferments which might be capable of attacking wood; more particularly of one which could hydrolyse the celluloses or hemi-celluloses of wood with the production of glucose.

The difficulty in these experiments was to obtain sufficient material. The "liver" of *Teredo* is a thin skin of tissue extending over the surface of the gonad and is difficult to free completely from the latter; moreover, a very large number of individuals are necessary in order to provide sufficient liver substance for a satisfactory experiment.

The first experiment tried in this direction was as follows. The livers of six *Teredo* were excised, and freed as completely as possible from intermixed gonad; the tissue was then quickly and thoroughly minced up and mixed with distilled water to which toluene was added to inhibit bacterial action. The

mixture was allowed to stand for 24 hours and then filtered through muslin; the extract of the liver thus obtained was used for the following experiments:

(1) Tube containing 0·16 g. filter paper (pure cellulose) + 8 cc. water + 2·5 cc. extract.
(2) The same boiled in order to destroy enzymes.
(3) 2·5 cc. starch solution + 2·5 cc. water + 2·5 cc. extract.
(4) Same, boiled.
(5) 0·2 g. fibrin + 5 cc. water + 2·5 cc. extract.
(6) Same, boiled.

In all cases toluene was added as an antiseptic.

The contents of tubes 1–4 were examined at intervals of 24 hours for reducing substances. At the end of 72 hours the contents of tube 3 gave a marked reduction with Benedict's reagent, and the iodine reaction had completely disappeared; on treatment with phenylhydrazine hydrochloride in presence of acetic acid, a plentiful crop of glucosazone crystals was obtained. The contents of tube 4 at the end of the same period gave no reduction with Benedict's sclution and still gave an intense iodine reaction.

The contents of tubes 1 and 2 gave no reduction with Benedict's solution even after a fortnight. No evidence of the digestion of the fibrin could be obtained from the contents of tubes 5 and 6. A second experiment was carried out in which the livers of 12 *Teredo* were excised and extracted as before, and the resulting extract (10 cc.) was divided into two equal portions, to each of which was added 5 cc. of water and 0·5 g. of finely sifted sawdust. One of these tubes was then boiled in order to destroy any enzymes which might be present. After keeping for a week, with toluene as an antiseptic, the contents of the unboiled tube gave a distinct reduction with Benedict's reagent, while those of the boiled tube gave a very slight reduction. Both solutions were then treated under the same conditions with phenylhydrazine hydrochloride and acetic acid, with the result that the contents of the unboiled tube gave a fairly large amount of glucosazone crystals, whereas those of the boiled tube only gave a few crystals of this substance.

Owing to the shortage of material and the consequent impossibility of running rigorous controls, it can hardly be claimed that the results are conclusive, except in the case of the starch digestion; here at least we have a clear demonstration of the fact that the liver contains an enzyme or enzymes which can hydrolyse starch completely to glucose.

In the case of the experiment with sawdust it might be argued that the possibility of the glucose having originated from the liver itself was not excluded. Any formation of glucose from the liver would probably, however, have taken place during the process of extraction, and this may account for the small quantity of glucose which was found in the control experiment. It seems difficult to account for the very much larger amount of glucose which was present in the unboiled tube, unless we assume it to have been formed from the wood by the action of an enzyme.

The failure, in the first experiment, to obtain any evidence of hydrolysis of cellulose is not such a contradiction of this hypothesis as at first appears. In the first place the liver extract used was very much less concentrated than that used on the wood and, secondly, there are present in wood hemi-celluloses which one would expect to be more readily hydrolysed than pure cellulose. Moreover, there is the further possibility that there may be other substances present in wood which are necessary as co-enzymes to the ferment in question.

Without more data it is impossible to draw definite conclusions, but the balance of evidence so far obtained is certainly in favour of the view that *Teredo* has in its liver an enzyme capable of producing glucose from some constituent of wood, and hence one may suppose that the wood, at least to this extent, is made use of as a source of nourishment.

It is hoped, by further experiments, to obtain more definite evidence upon this point.

The experiments described above form part of a research under the auspices of the Scientific and Industrial Research Department, undertaken on behalf of the Sea-Action Committee of the Institution of Civil Engineers, to whom I am indebted for permission to publish the results. I also wish to express my gratitude to Dr E. J. Allen, F.R.S., for assistance and advice, and to Dr J. S. Dunkerley for taking the photograph.

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